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April 26, 2005

Atty. Docket No. US-128O

In re application of: Kanno et al.
Application. No.: 09/868,338
Filing Date: June 18, 2001
Title: ABC TRANSPORTER AND GENE ENCODING SAME

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA. 22313-1450

Sir:
Transmitted herewith is an Appeal Brief in the above-identified application.

☒ The fee under 37 CFR 1.17(c) for filing a brief in support of an appeal is enclosed.
☐ The fee under 37 CFR 1.17(d) to request for oral hearing is enclosed.
☐ An extension of time petition and the requisite fee of for months is enclosed.

The above fees are submitted via the following payment method:

☒ Please charge Deposit Account No. 50-3077 in the amount of $500.00.
☐ A check in the amount is enclosed, including to cover any extension of time fee.
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- ☒ Deposit Account No. 50-3077
- ☐ the credit account identified in PTO-2038.
- ☒ Any filing fees under 37 C.F.R. § 1.16 for the presentation of extra claims.
- ☒ Any patent application processing fees under 37 C.F.R. § 1.17.
- ☒ Any Extension of Time fee.

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Respectfully submitted,

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Reg. No. 39,571

Date: April 26, 2005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Kanno et al.

Application No.: **09/868,338**

Filing Date: June 18, 2001

For: ABC TRANSPORTER AND GENE ENCODING SAME

Art Unit: 1646

Examiner: N. S. Basi

Attorney Ref. No.: US-128O

BRIEF FOR APPELLANT

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

COMES NOW the Appellant to present this Brief in support of the appeal of the final rejections of Claims 7, 15, and 16 in the above-captioned patent application. A petition for an extension of time is not necessary, as the Notice of Appeal was filed March 7, 2005, and this appeal is filed with 2 months of the filing of the Notice of Appeal in accordance with 37 C.F.R. §41.37(a)(1).

It is not believed that extensions of time are required, beyond those that may otherwise be provided for in accompanying documents. If, however, additional extensions of time are necessary to prevent abandonment of this application or dismissal of this appeal, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and the Commissioner is hereby authorized to charge fees necessitated by this paper, and to credit all refunds and overpayments, to deposit account 50-3077.

For the following reasons, Appellant respectfully submits that the final rejection of each of Claims 7, 15, and 16 in this application is in error, and therefore respectfully requests reversal of the rejections.

TABLE OF CONTENTS

I. Real Party in Interest

The real party in interest is Ajinomoto Co., Inc, a corporation of Japan.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of Claims

Claims 1-4, 7 and 9-16 are pending. Claims 7 and 15-16 stand finally rejected in the Final Rejection dated October 6, 2004, and reiterated in the Advisory Action dated 14 March 2005, and are on appeal. Claims 1-4 and 9-14 are withdrawn from consideration as being drawn to a non-elected invention.

IV. Status of Amendments

All amendments to the claims have been entered, including the amendment after final rejection filed on 04 January 2005.

V. Summary of Claimed Subject Matter

The claims are directed to an isolated DNA which comprises the nucleotide sequence from 1117 to 1725 of SEQ ID NO: 7 (see page 5, line 22 to page 6, line 18), and a protein encoded by this sequence or having the L-amino acid sequence of SEQ ID NO:9 (see page 3, lines 21 to page 4, line 3). This protein is a novel ABC transporter. The gene which encodes this novel ABC transporter can be utilized for breeding of a microorganism to modify transport of L-amino acids across the membrane (see page 7, lines 6-14, and table 1).

VI. Grounds of Rejection to be Reviewed on Appeal

A. Whether Claims 7 and 15-16 are unpatentable under 35 U.S.C. §101 for lack either a specific or substantial asserted utility, or a well-established utility.

B. Whether Claims 7 and 15-16 are unpatentable under 35 U.S.C. §112, 1st

paragraph, for a lack of enablement by the specification concomitant with the scope of the claims.

VII. Argument

In the Office Action dated 06 October 2004 ("the final rejection"), beginning at page 2, Claims 7, 15, and 16 were rejected under 35 U.S.C. §101 and 35 U.S.C. §112, 1st paragraph, for allegedly failing to provide a specific and substantial utility, and for failing to be supported by a specification that includes an enabling disclosure. For at least the following reason, these rejections are in error and should be reversed.

A. Legal Standard Required by 35 U.S.C §101 and 35 U.S.C. §112, 1st paragraph

Patent appellants are required to assert a utility in the specification for the claimed invention under 35 U.S.C. §101, and this requirement has been interpreted by the Courts to have two prongs. First, the invention must be operable, which means it must be "capable of being used to effect the object proposed" in the specification. See *Mitchell v. Tilghman*, 86 U.S. 287, 396 (1873). This standard has been construed by the Federal Circuit as a minimal threshold, in that "when a properly claimed invention meets at least one stated objective, utility under Section 101 is clearly shown." See *Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 958 (Fed. Cir. 1983). In fact, to hold claims invalid under Section 101, it must be shown that the invention is "totally incapable of achieving a useful result." See *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555 (Fed. Cir. 1992). In fact, inoperable embodiments are permitted. *Atlas Powder co. v. E.I. du Pont De Nemours & Co.*, 750 F.2d 1569, 1576 (Fed. Cir. 1984).

The second prong of the requirement is that the invention must possess a practical utility, in that a "specific benefit exists in currently available form." See *Brenner v. Manson*, 383 U.S. 519, 534-35 (1966). The Supreme Court stated in *Brenner* that no patent could be granted "on a chemical compound whose sole 'utility' consists of its potential role as an object of use-testing" However, there is no requirement for a "substantial" utility, nor a "well-established" utility.

In addition to the utility requirement, the first paragraph of 35 U.S.C. §112 requires that

the specification describe the invention sufficient to enable any person skilled in the art to which it pertains to make and use the claimed invention. The focus of the statute is on the person skilled in the art rather than the general public, and enablement is determined with reference to the knowledge possessed by this hypothetical artisan. The test for this standard is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art, without undue experimentation. *United States v. Telectronics, Inc.*, 857 F.2d 778, 785 (Fed. Cir. 1988). The seminal case in determining if a claim meets this standard, *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988), promulgated a series of factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is 'undue.'

B. The rejection of Claims 7, 15, and 16 under 35 U.S.C. §101 is in error

Claims 7, 15, and 16 were rejected under 35 U.S.C. §101, as allegedly not being supported by a specific and substantial asserted utility or a well-established utility. Appellants assert that the Examiner has not met his initial burden of challenging appellant's presumptively correct assertion of utility. See *In re Swartz* 232 F.3d 862 (Fed. Cir. 2000). The burden of rebuttal only shifts to appellants once the Examiner provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility. *Id.* See also *In re Brana* 51 F.3d 1560 (Fed. Cir. 1995). The Examiner has stated that appellants have failed to provide a "specific utility", a "substantial utility" or a "well established" utility (see First Office Action of January 14, 2004, page 4). The Examiner also asserts there is no "real world" use for the claimed invention (see First Office Action of January 14, 2004, page 6).

The Examiner has alleged throughout the prosecution that the asserted utility is for "treating an unspecified, undisclosed disease or condition" (see Office Action of January 14, 2004, pages 5-6, and the Final Rejection of October 6, 2004, page 6). This assertion is entirely false as appellants do not state anywhere or suggest, either explicitly or inherently, that this is the asserted utility. The asserted utility of the claimed gene and protein of the present invention is

that they are useful for breeding of a microorganism for the purpose of modifying transport of L-amino acids across a cell membrane (see instant specification at page 1, lines 9-12, page 3, lines 3-7, page 8, table 1, and page 21, lines 15-19). This has nothing to do with treating a disease or condition. If the Examiner has not understood the asserted utility, he certainly cannot meet his initial burden of providing evidence to establish reasonable doubt, and thereby shifting the burden to appellants. For this reason alone, the rejection should be withdrawn.

The Examiner has injected requirements and standards into 35 U.S.C. §101 that simply do not exist. Furthermore, the Examiner has elevated the requirements of 35 U.S.C. §101 and §112, 1st paragraph to mean that appellants must provide clinical utility for a biological invention. There is no requirement in the statute or as interpreted by the Courts that the utility be "substantial" or "well-established", nor is there any requirement that a biological invention must, *de facto*, demonstrate clinical usefulness. The seminal case, *Brenner v. Manson* 383 U.S. 519 (1966), did state that to satisfy §101, the disclosure must assert a "specific benefit... in currently available form" and was cited by the Federal Circuit in reference to an asserted utility of a chemical compound as "a potential role as an object of use testing". See *Zeigler*. However, there is no requirement for a "substantial" utility, nor a "well-established" utility.

The asserted utility of the claimed gene and protein of the present invention is that they are useful for breeding of a microorganism for the purpose of modifying transport of L-amino acids across a cell membrane (see the specification at page 1, lines 9-12, page 3, lines 3-7, page 8, table 1, and page 21, lines 15-19). This is applicant's asserted utility for the claimed invention. The Examiner actually did challenge this statement in the Final Rejection of October 6, 2004 by continuing to argue that a "well-established utility" has not been established, and cited to a reference which allegedly teaches that over 50 ABC transporters are known and specificities are varied and diverse. Actually, appellants assert that such a disclosure in a publicly available document merely bolsters the argument that the invention has a specific and substantial utility, since appellants have shown that the novel gene and protein claimed is an ABC transporter, and this class of compounds is well-established class of compounds with a specific function in the cell machinery, *i.e.* that of facilitating ligands into and out of the cell.

The ABC transporters have an established physiological function of uptake and excretion of substances into and out of the cell, hence the term 'transporters'. This is obviously an important and defined function in the cell machinery, allowing a cell to, *e.g,*. excrete toxic and unneeded substances, while importing useful substances for its metabolism. Appellants assert that the Examiner has failed to establish reasonable doubt of the objective truth of any of the above statements, and the Examiner has provided absolutely no evidence to support his otherwise bald assertions.

The gene/protein of the present invention has several asserted utilities: the transport of L-amino acids across the membrane of the cell, for secreting L-amino acids out of the cell, and for importing L-amino acids into the cell. Transporters have a defined and credible usefulness which is practical, in that these proteins can be expressed in a cell and effect the transport of substances, and in the instant invention, L-amino acids, inside and outside of the cell. Any person of ordinary skill in the art would recognize this utility as useful and 'in currently available form' and not merely an object of further 'use-testing'. The protein of the instant invention is clearly an ABC transporter, and this utility has been established by the inventors. No further use-testing needs to be conducted to establish utility. Although further research might be conducted to further evaluate the protein, the current evidence as presented is sufficient to satisfy Section 101, since it shows the claimed gene/protein's usefulness as a transporter in the cell machinery.

To further establish the claimed gene/protein as a member of the ABC transporter family, appellants provided to the Examiner a FASTA search of the protein of SEQ ID NO: 9 in the amendment submitted on July 9, 2004. In this search, the only matches which were found to have significant homology were ABC transporters. This homology clearly establishes the claimed protein as a member of this family.

As further evidence to rebut the Examiner's assertions, appellants provided a reference (*Arch Microbiol* 180:88-100 (2003)) in response to the First Office Action which demonstrates the known usefulness of ABC transporters as L-amino acid transporters, involved in the uptake and excretion. Further references, EP1038970 and AU 199719218, show that production of an L-

amino acid can be effected by disrupting a gene which encodes an L-amino acid uptake protein, or that amplifying a gene involved in L-amino acid export can enhance the production of an L-amino acid. Therefore, further usefulness of the ABC transporter gene/protein is established.

The Examiner has also asserted that appellants have failed to disclose ligands or compounds which can be transported, and therefore, the asserted utility for the ABC transporters are "essentially methods of treating unspecified, undisclosed diseases or conditions, which does not define a 'real world' context of use." See the First Office action of January 14, 2004, page 6. First, appellants have clearly stated that the asserted utility of the novel ABC transporter gene/protein is for transporting L-amino acids in/out of the cell. Second, the Examiner's statements demand the question: why must the asserted utility have a therapeutic use? Appellants do not assert that the claimed invention is useful for treatment of diseases or conditions. In fact, as anyone of ordinary skill in the art would know, L-amino acid production using bacteria is a large multi-billion dollar business in the United States and around the world. Bacterial breeding methods for increasing L-amino acid production are the research focus of many international corporations, including the assignee of the present application. Establishing new bacteria which can efficiently produce L-amino acids is also an area of intense research interest for many of these same companies. L-amino acids have many 'real world' applications, only a few of which are to treat 'diseases or conditions'. In fact, one of the most common uses for L-amino acid is for feed supplements for livestock. The Examiner cannot require proof of pharmaceutical utility, particular where none has been asserted. Again, the Examiner has failed to establish reasonable doubt of applicant's asserted utility, which has nothing to do with treatment of any disease or condition.

On page 9 of the First Office Action of January 14, 2004, the Examiner again tries to require evidence of pharmaceutical usefulness in stating that "There is no evidence or record or any line of reasoning that would support a conclusion that the claimed protein/polynucleotide was, as of the filing date, useful for diagnosis, prevention and treatment of an [*sic*] disease, or for screening compounds." On page 11, the Examiner asserts "The assertion that the claimed invention has utility in drug screening, testing, drug development and disease diagnosis, do not

meet the standards for a specific substantial or well-established utility....". There is no instance in the specification where appellants assert the claimed invention has utility in drug screening, testing, drug development, or disease diagnosis. Appellants cannot be required to provide evidence for a utility which has not been asserted or is counter to the utility that has been asserted. The gene/protein of the present invention is useful, in one instance, as a transporter for L-amino acids to the outside of the cell membrane. The Examiner has failed to establish reasonable doubt that the claimed gene/protein is not useful as asserted by appellants. The gene/protein is clearly a member of the ABC transporter family of proteins. This family of proteins is clearly involved in the uptake and secretion of L-amino acids in bacterial cells. Finally, it is clear that production of an L-amino acid can be enhanced by disrupting a gene involved in uptake of L-amino acids or amplification of a gene involved in secretion of an L-amino acid. These utilities are involved in L-amino acid production, not diagnosing, preventing, and/or treating a disease, as the Examiner is asserting.

The Examiner asserts on page 11 of the First Office Action of January 14, 2004 that appellants have failed to show whether an increase in expression of the claimed gene/protein would be toxic. Toxicity testing is not required to show utility, particularly when the asserted utility is not pharmaceutical in nature. Such a requirement is entirely unfounded.

The Examiner has further based the rejection on the assertion that the substance to be transported has not been identified, nor has the transporter been identified as an 'exporter' or 'importer'. To the contrary, there are multiple instances whereby appellants assert in their specification that the transporter is effective for exporting of L-amino acids for use in bacterial fermentative production of said L-amino acids. See *e.g.,* page 3, lines 3-7, page 7, lines 6-14, and table 1 on page 8. Appellants clearly state that the asserted utility of the novel ABC transporter gene/protein is for transporting L-amino acids in/out of the cell.

The Examiner has repeatedly asserted that the facts presented in this application are directly analogous to that which was addressed in *Brenner v. Manson.* Appellants assert this is not the case at all. In fact, *Brenner* addressed the situation where the specification failed to disclose **any utility** for the compounds produced by the process, but merely relied on the fact

that it was well-known in the art that steroids of a class that included the products were undergoing screening for possible tumor-inhibiting effects in mice, and that an adjacent homolog of the product at issue was known to be effective in this application. This is at least one step removed from the situation from the facts of the present invention. First, the present specification discloses and asserts a credible utility for the product of the present invention. No screening or further assessment is necessary. Furthermore, whereas in *Brenner*, the utility had to do with tumor inhibition in mice, the asserted utility of the present invention concerns bacterial cell transport. Such a utility involves simpler cells and simpler processes, which therefore, should require a lower standard of operability and lesser skill in the art. For these reasons, appellants assert that comparison of the facts of *Brenner* with the current specification is unfounded.

For this reason, appellants assert that the Examiner has failed to establish reasonable doubt as to the operability or practical utility of the claimed invention, particularly since the Examiner does not appear to understand or comprehend the asserted utility. For these reasons, appellants respectfully request the rejection be withdrawn.

C. The rejection of Claims 7, 15, and 16 under 35 U.S.C. §112, 1st paragraph, enablement, is in error

Claims 7, 15, and 16 are rejected under 35 U.S.C. §112, 1st paragraph for not being enabled by the specification. The Examiner alleges that a person of skill in the art would not know how to use the claimed invention based on appellant's specification. Again, the Examiner asserts that the person of skill in the art would not know how to use the invention since no specific function has been disclosed for the claimed polynucleotide. As stated above, the claimed gene/protein has been identified as a member of the ABC transporter family, members of which are useful as transporters of L-amino acids outside of the cell (see evidence and arguments presented above). This is a 'real world' utility since L-amino acid production is a multi-billion dollar business.

The Examiner has argued the rejections under 35 U.S.C. §101 and §112, 1st paragraph

together, and therefore appellants do not further address the rejection under 35 U.S.C. §112, 1st paragraph, as the above arguments sufficiently address the 'how to use' requirement of the statute.

For at least the foregoing reasons, appellants assert that the rejections should be withdrawn and the claims passed to issue.

X. Conclusion

For at least the foregoing reasons, Appellant respectfully submits that the subject matters of Claims 7 and 15-16, each taken as a whole, are patentable. Accordingly, Appellant respectfully submit that the rejections thereof are in error and requests reversal of the rejections of Claims 7, 15, and 16 under sections 101 and 112, 1st paragraph.

Respectfully submitted,

By: 

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Date:

APPENDIX: CLAIMS ON APPEAL

7. An isolated DNA comprising the nucleotide sequence of nucleotide number 1117 to 1725 of SEQ ID NO: 7.

15. An isolated protein encoded by the DNA of claim 7.

16. An isolated protein of claim 15, wherein said protein has the amino acid sequence of SEQ ID NO: 9.

EVIDENCE APPENDIX

The following four references were submitted to the Examiner in response to the First Office Action. The Examiner considered these in the Final Rejection dated October 6, 2004:

A) FASTA Search Results

B) Zhang et al., *Arch. Microbiol.* 180:88-100 (2003)

C) EP 1 038 970 A2

D) AU 1997 19218 B2

FASTA Search Result

The Reference 1

Computed at GenomeNet FASTA Server (Kyoto Center) on Wed Jun 2 20:04:54 JST 2004

Database Name NR-AA
>query
1MIEINDLKK SFGVRILWQG LSHKFLPGTM TALTGASGSG KSTLLNCLGT
L51DKPSSGQ ILVEDVDLLK LSTRKQRLYR KNTVGYLFQD YALIPDRTVK
FNL101QLAV EKHKWPEIPQ VLHAVGLESF EEKPVFELSG GEQQRTALAR
VLLKNP151R IILADEPTGA LDLTNSELVI EALRALADKG ATVVVATHSP
LFRESADTI2 011KL

WARNING: possibly wrong combination
command: fasta
query: (Not FASTA Format)
database: nr-aa (Prot)

command	query	database
fasta	Prot	Prot
	Nucl	Nucl
tfasta	Prot	Nucl

FASTA searches a protein or DNA sequence data bank
version 3.4t10 Dec 12, 2001
Please cite:
W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

/bio/local/WWW/pub/tmp/fasta.68432364/fasta.tmp: 203 aa
>query
vs /bio/db/blast/db/nr-aa library
searching /bio/db/blast/db/nr-aa library

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       opt      E()
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  22     5     0:=          one = represents 2646 library sequences
  24    25     1:*
  26   111    32:*
  28   804   349:*
  30  3193  2122:*=
  32  9990  8204:===*
  34 24842 22248:========*=
  36 48798 45692:==================*=
  38 76872 75511:============================*=
  40 111112 105331:=======================================*==
  42 142638 128755:=================================================*=====
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  46 158731 144659:=======================================================*=====
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  52 108134 111106:=========================================*
  54 89128 94904:================================== *
  56 72856 79274:============================= *
  58 57683 65083:======================  *
  60 45073 52721:================== *
  62 35897 42266:============== *
  64 28047 33614:=========== *
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  68 16380 20898:=======*
  70 12373 16377:===== *
  72  9488 12797:====*
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  84  1893  2840:=*
  86  1362  2197:*
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  90   742  1316:*
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  94   474   788:*         :===*
  96   359   609:*         :==*
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 118    48    36:*         :*
>120 11700    28:*====     :*====================================
500240239 residues in 1551842 sequences
 statistics extrapolated from 60000 to 1540015 sequences
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 mean_var=74.8953+/-16.395, 0's: 157 Z-trim: 407  B-trim: 2437 in 2/63
 Lambda= 0.1482
 Kolmogorov-Smirnov  statistic: 0.0444 (N=29) at  50

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 join: 36, opt: 24, open/ext: -10/-2, width:  16
 Scan time: 267.910
The best scores are:                                        opt bits E(1551842)
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Top 10 Clear Select operation Exec

☑	pir:G95079 [G95079] ABC transporter, ATP-binding	(213)	524	120	3.6e-26
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☑	pir:B97947 [B97947] hypothetical protein ABC-NBD	(213)	520	120	6.5e-26
☑	prf:2805303ELE ABC transporter - Clostridium perf	(211)	519	119	7.5e-26
☑	tr:Q892J8 [Q892J8] Transporter. >gp:AE015943_111 [	(212)	514	118	1.6e-25
☑	pir:E95232 [E95232] ABC transporter, ATP-binding	(213)	513	118	1.8e-25
☑	pir:G95228 [G95228] ABC transporter, ATP-binding	(210)	492	114	4.1e-24
☑	prf:2713501E ATP-binding protein - Lactococcus la	(207)	490	113	5.4e-24
☑	pir:A95013 [A95013] hypothetical protein SP0111 [	(213)	487	113	8.6e-24
☑	pir:D97884 [D97884] hypothetical protein ABC-NBD	(213)	486	112	1e-23
☐	prf:2724351JF ABC transporter - Streptomyces aver	(248)	480	111	2.7e-23
☐	pir:B86626 [B86626] ABC transporter ATP-binding p	(211)	474	110	5.9e-23
☐	pir:D69433 [D69433] ABC transporter, ATP-binding	(226)	466	108	2e-22
☐	sp:Y065_MYCPN [P75612] Hypothetical ABC transport	(465)	466	108	3.3e-22
☐	tr:Q9L0J9 [Q9L0J9] Putative ABC-transporter ATP-b	(246)	463	108	3.3e-22
☐	pir:B69377 [B69377] ABC transporter, ATP-binding	(228)	461	107	4.3e-22
☐	prf:2719186AYG ABC transporter - Sulfolobus tokod	(232)	460	107	5e-22
☐	prf:2813338EKK MWP018 gene - Staphylococcus aureu	(208)	459	107	5.4e-22
☐	tr:Q8P0U2 [Q8P0U2] Putative ABC transporter (ATP-	(233)	459	107	5.8e-22
☐	pir:A84088 [A84088] ABC transporter (ATP-binding	(228)	458	106	6.7e-22
☐	pir:B97087 [B97087] ABC-type transport system, AT	(255)	457	106	8.3e-22

tr:Q8RCC2 [Q8RCC2] ABC-type polar amino acid tran	(240)	408	96	1.1e-18
pir:AH1709 [AH1709] ABC transporter (ATP-binding	(255)	408	96	1.2e-18
pir:D97973 [D97973] hypothetical protein ABC-NBD	(271)	408	96	1.2e-18
tr:Q7P537 [Q7P537] ABC transporter ATP-binding pr	(220)	407	95	1.2e-18
tr:Q9CM47 [Q9CM47] Hypothetical protein PM0996.>g	(649)	412	97	1.3e-18
tr:Q88F88 [Q88F88] ABC export system, permease/AT	(654)	412	97	1.3e-18
tr:Q81K43 [Q81K43] ABC transporter, ATP-binding p	(226)	407	96	1.3e-18
trnew:AAR35564 [AAR35564] ABC transporter, ATP-bi	(232)	407	96	1.3e-18
tr:Q8PYB3 [Q8PYB3] ABC transporter, ATP-binding p	(293)	408	96	1.3e-18
sp:BCEA_BACSU [O34697] Bacitracin export ATP-bind	(253)	407	96	1.4e-18
pir:AB1339 [AB1339] ABC transporter (ATP-binding	(255)	407	96	1.4e-18
prf:2805303ARP ABC transporter - Clostridium perf	(255)	407	96	1.4e-18
tr:Q92NU9 [Q92NU9] Probable transmembrane ATP-bin	(647)	411	97	1.5e-18
pir:D69858 [D69858] ABC transporter (ATP-binding	(230)	406	95	1.5e-18
pir:G96929 [G96929] ABC transporter ATP-binding p	(238)	406	95	1.5e-18
tr:Q8TQ83 [Q8TQ83] Lipoprotein releasing system	(227)	405	95	1.7e-18
pir:D69627 [D69627] cell-division ATP-binding pro	(228)	405	95	1.7e-18
pir:B86714 [B86714] hypothetical protein yhcA [im	(664)	410	97	1.7e-18
trnew:AAR34717 [AAR34717] ABC transporter, ATP-bi	(234)	405	95	1.7e-18
prf:2824301B bacitracin resistance-related protei	(250)	405	95	1.8e-18
trnew:AAT04916 [AAT04916] ABC transporter, ATP-bi	(255)	405	95	1.9e-18
tr:Q8G5S1 [Q8G5S1] Possible ATP binding protein o	(263)	405	95	1.9e-18
pir:T36431 [T36431] probable ABC-type transport s	(264)	405	95	1.9e-18
trnew:CAE50415 [CAE50415] Putative ABC transport	(249)	404	95	2.1e-18
tr:Q8DUD2 [Q8DUD2] Putative ABC transporter, ATP-	(250)	404	95	2.1e-18
tr:Q8PFI5 [Q8PFI5] ABC transporter ATP-binding pr	(229)	403	95	2.3e-18
sp:LOLD_ECOL6 [Q8FIM7] Lipoprotein releasing syst	(233)	403	95	2.3e-18
sp:LOLD_SHIFL [Q83RS0] Lipoprotein releasing syst	(233)	403	95	2.3e-18
trnew:CAF30191 [CAF30191] ABC transporter:ATPase	(241)	403	95	2.4e-18
pir:A83744 [A83744] ABC transporter (ATP-binding	(260)	403	95	2.5e-18
prf:2901400RM ABC transporter - Mycoplasma penetr	(328)	404	95	2.6e-18
pir:B97146 [B97146] ABC-type transport system, AT	(224)	402	94	2.6e-18
tr:Q884D4 [Q884D4] Macrolide ABC efflux protein.>	(656)	407	96	2.7e-18
tr:Q8R8L8 [Q8R8L8] Predicted ATPase involved in c	(228)	402	94	2.7e-18
prf:2824433GH ABC transporter - Oceanobacillus ih	(228)	402	94	2.7e-18

>>pir:G95079 [G95079] ABC transporter, ATP-binding prote (213 aa)
initn: 438 init1: 284 opt: 524 Z-score: 613.5 bits: 120.5 E0: 3.6e-26
Smith-Waterman score: 524; 41.905% identity (44.000% ungapped) in 210 aa overlap (1-203:1-207)

```
               10        20        30        40        50        60
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQILV
       :::.....:.:: : :.. .. . :  :. :: :: :::: . : :.  ..:::.
pir:G9 MIELKNISKKFGSRQLFSDMNLHFEGGKIYALIGTSGCGKTTLLNMIGRLEPYDKGQIIY
               10        20        30        40        50        60

               70        80        90       100              110
query  EDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAV-------EKHKWPEIPQ
       . ...:  .   :  .. .. . :::::.. :: .. ::: ::.:..          ::.:   . :
pir:G9 DGTSLKDI---KPSVFFRDYLGYLFQDFGLIESQTVKENLNLGLVGKKLKEKEKISLMKQ
                 70        80        90       100       110

          120       130       140       150       160       170
```

ABC-Tranpoter.

```
query  VLHAVGLESFE-EKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIEA
       .:. :.: .. ..:.:::::::: ::.:::.. ::.: .:::::::.. ::  ::: ..
pir:G9 ALNRVNLSYLDLKQPIFELSGGEAQRVALAKIILKDPPLILADEPTASLDPKNSEELLSI
       120       130       140       150       160       170

           180       190       200
query  LRALADKGATVVVATHSPLFRESADTIIKL
       :..: . . :...:::.::  :..: . :..
pir:G9 LESLKNPNRTIIIATHNPLIWEQVDQVIRVTDLSHR
       180       190       200       210

>>prf:2504343R ORF - Streptococcus pneumoniae>tr:Q9ZHB1    (213 aa)
 initn: 434 init1: 284 opt: 521  Z-score: 610.0  bits: 119.9 E(): 5.6e-26
Smith-Waterman score: 521;  41.905% identity (44.000% ungapped) in 210 aa overlap (1-203:1-207)

               10        20        30        40        50        60
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQILV
       :::.....:.:: : :..  . .:  : .  :: :.:: :::.:::: . : :.  ..:::.
prf:25 MIELKNISKKFGSRQLFSDTNLHFEGGKIYALIGTSGCGKTTLLNMIGRLEPYDKGQIIY
               10        20        30        40        50        60

               70        80        90        100             110
query  EDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAV------EKHKWPEIPQ
       . ..:  .   :. .. .. .:::::. .:: ..::: :: :..       ::.:   . :
prf:25 DGTSLKDI---KSSVFFRDYLGYLFQDFGLIESQTVKENLNLGLVGKKLKEKEKISLMKQ
                 70        80        90        100       110

          120       130       140       150       160       170
query  VLHAVGLESFE-EKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIEA
       .:. :.: .. ..:.:::::::: ::.:::.. ::.: .:::::::.. ::  ::: ..
prf:25 ALNRVNLSYLDLKQPIFELSGGEAQRVALAKIILKDPPLILADEPTASLDPKNSEELLSI
       120       130       140       150       160       170

           180       190       200
query  LRALADKGATVVVATHSPLFRESADTIIKL
       :..: . . :...:::.::  :..: . :..
prf:25 LESLKNPNRTIIIATHNPLIWEQVDQVIRVTDLSHR
       180       190       200       210

>>pir:B97947 [B97947] hypothetical protein ABC-NBD [impo  (213 aa)
 initn: 434 init1: 284 opt: 520  Z-score: 608.9  bits: 119.6 E(): 6.5e-26
Smith-Waterman score: 520;  41.905% identity (44.000% ungapped) in 210 aa overlap (1-203:1-207)

               10        20        30        40        50        60
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQILV
       :::.....:.:: : :..  . .:  : .  :: :.:: :::.:::: . : :.  ..:::.
pir:B9 MIELKNISKKFGSRQLFSDTNLHFEGGKIYALIGTSGCGKTTLLNMIGRLEPYDKGQIIY
               10        20        30        40        50        60

               70        80        90        100             110
query  EDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAV------EKHKWPEIPQ
       . ..:  .   :. .. .. .:::::. .:: ..::: :: :..       ::.:   . :
pir:B9 DGTSLKDI---KPSVFFRDYLGYLFQDFGLIESQTVKENLNLGLVGKKLKEKEKISLMKQ
                 70        80        90        100       110

          120       130       140       150       160       170
query  VLHAVGLESFE-EKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIEA
       .:. :.: .. ..:.:::::::: ::.:::.. ::.: .:::::::.. ::  ::: ..
pir:B9 ALNRVNLSYLDLKQPIFELSGGEAQRVALAKIILKDPPLILADEPTASLDPKNSEELLSI
       120       130       140       150       160       170

           180       190       200
query  LRALADKGATVVVATHSPLFRESADTIIKL
       :..: . . :...:::.::  :..: . :..
pir:B9 LESLKNPNRTIIIATHNPLIWEQVDQVIRVTDLSHR
```

?

ABC Tranpoter

```
        180       190       200       210

>>prf:2805303ELE ABC transporter - Clostridium perfringe   (211 aa)
 initn: 539 init1: 289 opt: 519  Z-score: 607.7  bits: 119.4 E(): 7.5e-26
Smith-Waterman score: 519;  40.191% identity (41.379% ungapped) in 209 aa overlap (1-203:3-211)

                10        20        30        40        50
query     MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQI
           .::::.:.:..  .....  .: ..   : : :.: :: :::::: .: ..: . ::. :
prf:28 MNIIEISNLNKKYFDKVIFKDFSLSIKKGEMIAISGRSGCGKSTLLNMIGLIEKFDSGEI
               10        20        30        40        50        60

      60        70        80        90       100            110
query  LVEDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAVE-------KHKWPEI
       ... :  . :...    . .. ..::::..::. ..::. ::.::..       :.   ::
prf:28 IIDGVKNIKINSKLANKFLREKISYLFQNFALVDEETVEENLRLAIKHTIKNTKKIEEEI
               70        80        90       100       110       120

          120       130       140       150       160       170
query  PQVLHAVGLESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIE
       . :. ::::. ... ..:::::::::.:.::.:: .::::::::.::  : . . :
prf:28 IRCLKFVGLEGCQKNYIYELSGGEQQRVAIARLMLKPSEIILADEPTGSLDEENRDIIIS
              130       140       150       160       170       180

          180       190       200
query  ALRALADKGATVVVATHSPLFRESADTIIKL
       :. : ..: :.... ::.     ..:: :: :
prf:28 LLKELNESGKTIIIVTHDNYVAKQADRIIFL
              190       200       210

>>tr:Q892J8 [Q892J8] Transporter.>gp:AE015943_111 [AE015   (212 aa)
 initn: 282 init1: 282 opt: 514  Z-score: 601.9  bits: 118.4 E(): 1.6e-25
Smith-Waterman score: 514;  43.077% identity (44.444% ungapped) in 195 aa overlap (1-189:3-197)

                10        20        30        40        50
query     MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQI
         .......  :.:: .:. ...:  ..   : .  :.:::::::::.:: .: :.   .::..
tr:Q89 MSIVKMENITKKFGDKIILNNFSLDIQDGELLAVTGASGSGKSTILNIIGLLEGFDSGKL
               10        20        30        40        50        60

      60        70        80        90       100          110
query  LVEDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLA---VEKHKWPE---I
       ...   . .:... :.      .. .:::::..::. ..:: .::.::   :.:.:  .   :
tr:Q89 ILDGDENIKINSSKSNKILREKIGYLFQNFALVDEETVYYNLHLALKYVKKNKKEKDELI
               70        80        90       100       110       120

          120       130       140       150       160       170
query  PQVLHAVGLESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIE
       .::. ..::.:.. .::::::::::...::.:::  .:::::::::.::  : .::.
tr:Q89 KNVLKQMNLEGYEKRKIFELSGGEQQRVSIARLLLKPSKIILADEPTGSLDAKNRDLVLY
              130       140       150       160       170       180

          180       190       200
query  ALRALADKGATVVVATHSPLFRESADTIIKL
       :  :  .: ::.:.::.
tr:Q89 YLNKLNKEGKTVIVVTHDMEVAKKCHRTISLN
              190       200       210

>>pir:E95232 [E95232] ABC transporter, ATP-binding prote   (213 aa)
 initn: 445 init1: 266 opt: 513  Z-score: 600.8  bits: 118.1 E(): 1.8e-25
Smith-Waterman score: 513;  42.857% identity (45.000% ungapped) in 210 aa overlap (1-203:1-207)

              10        20        30        40        50        60
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQILV
       ::.:. :.:.:. :....::. :.   : .  :: : :::::.:::: :: :.: ..:..:
```

Tranpoter

ABC Tranporter

```
pir:E9 MIDIQGLEKKFNDRAIFSGLNLKLEKGKVYALIGKSGSGKTTLLNILGKLEKIDGGRVLY
               10        20        30        40        50        60

               70        80        90       100       110       120
query  EDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAVEKHKWPEIPQVLHAVG
       .  ::  . ::.   : .. .:::::.. :. .... : :: :.   .: .. .. . ::
pir:E9 QGKDLKTIPTRE---YFRDQMGYLFQNFGLLENQSIKENLDLGFVGQKISKVERLERQVG
               70        80        90       100       110

                    130       140       150       160       170
query  -LESFE------EKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIEA
        ::. .      :. .. ::::: :: :::.. :::: . :::::::. :::  ::: :..
pir:E9 ALEKVNLGYLDLEQKIYTLSGGEAQRVALAKTILKNPPLILADEPTAALDPENSEEVMNL
       120       130       140       150       160       170

          180       190       200
query  LRALADKGATVVVATHSPLFRESADTIIKL
       : : :..  ...:::. ::  .. :: :: .
pir:E9 LVDLKDENRIIIIATHNPLVWNKADEIIDMRKLAHV
       180       190       200       210
```

>>pir:G95228 [G95228] ABC transporter, ATP-binding prote (210 aa)
initn: 500 init1: 270 opt: 492 Z-score: 576.6 bits: 113.6 E(): 4.1e-24
Smith-Waterman score: 492; 40.952% identity (43.216% ungapped) in 210 aa overlap (1-203:1-206)

```
               10        20        30        40        50        60
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQILV
       ::..... :::: : :... ::  :  : . :: :. ::::: :: . : :. : . : :.
pir:G9 MIELKQVSKSFGERELFSNLSMTFEAGKVYALIGSSGSGKTTLMNMIGKLE-PYDGTIFY
               10        20        30        40        50

               70        80        90       100       110
query  EDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAVEKHKWPEIP-----Q
       .  ::   .. :.  . .. .:::::... :: ..... :: :.   .: .        :
pir:G9 RGKDL---ANYKSSDFFRHELGYLFQNFGLIENQSIEENLKLGLIGQKLSRSEQRLRQKQ
      60        70        80        90       100       110

          120       130       140       150       160       170
query  VLHAVGLESFE-EKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIEA
       . :. :::  .. . : . :::::: :: :::.. :::: . :::::::.. : .. :. :. :
pir:G9 ALEQVGLVYLDLDKRIFELSGGESQRVALAKIILKNPPFILADEPTASIDPATSQLIMEI
       120       130       140       150       160       170

          180       190       200
query  LRALADKGATVVVATHSPLFRESADTIIKL
       : . : :.  ...:::. : . : :: .. .
pir:G9 LLSLRDDNRLIIIATHNPAIWEMADEVFTMDHLK
       180       190       200       210
```

ABC Transporter

>>prf:2713501E ATP-binding protein - Lactococcus lactis (207 aa)
initn: 422 init1: 236 opt: 490 Z-score: 574.3 bits: 113.2 E(): 5.4e-24
Smith-Waterman score: 490; 41.905% identity (44.000% ungapped) in 210 aa overlap (1-203:1-207)

```
               10        20        30        40        50        60
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQILV
       :::..... ::   . . ..... :   .  ::. : ::::. :::: . : :.  :. :. ::.
prf:27 MIELKNIEKSYDNHNILHNFNYQFKDNKSYALVGKSGSGKTTLLNIIGRLELPDKGDILI
               10        20        30        40        50        60

               70        80        90       100           110
query  EDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAV----EKHKWPEI--PQ
       .: : ::  :  . : : :. .:::::. :. :: .... : ::. ::       :..  ::  .
prf:27 DD-DNLK--TIPERRYFKDYLGYLFQNYGLIDNESIKDNLKLAFIGKKLKNQDQEIIMSK
                70        80        90       100       110
```

ATP - ABC Transpoter

```
            120         130         140         150         160         170
query   VLHAVGLESFE-EKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIEA
        .:  ::::... .. . : ::::: ::.:.:....:.: :::::::::.::  ...  :..

prf:27  ALSKVGLENYNIDRKIFSLSGGEAQRVAIAKLIIKSPPIILADEPTGSLDRETGKEVMDI
        120        130        140        150        160        170

             180         190         200
query   LRALADKGATVVVATHSPLFRESADTIIKL
        : .:. ...::..:::.     . .:.::.:

prf:27  LLSLVKENTTVIIATHDSHVYNRVDSIINL
        180        190        200
```

>>pir:A95013 [A95013] hypothetical protein SP0111 [impor (213 aa)
initn: 263 init1: 263 opt: 487 Z-score: 570.7 bits: 112.6 E(): 8.6e-24
Smith-Waterman score: 487; 40.191% identity (41.379% ungapped) in 209 aa overlap (1-203:1-209)

```
                10         20         30         40         50         60
query   MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQILV
        :::... :..: ... ..::..   : ..:..: ::::::::::: ::.:      ::.  .

pir:A9  MIELKNITKTIGGKVILDNLSLRIDQGDLVAIVGKSGSGKSTLLNLLGLIDGDYSGRYEI
                10         20         30         40         50         60

                70         80         90        100        110
query   EDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAVEKHKWPE-------IPQ
         :...  :..    .. ..::::.:::  :.::..::.::..    : :.         . .

pir:A9  FGQTNLAVNSAKSQTIIREHISYLFQNFALIDDETVEYNLMLALKYVKLPKKDKLKKVEE
                70         80         90        100        110        120

           120         130         140         150         160         170
query   VLHAVGLESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIEAL
        .:.  :::.   ..  : :::::::: :.::..::  ..::::::::.::   : .::.. :

pir:A9  ILERVGLSATLHQRVSELSGGEQQRIAVARAILKPSQLILADEPTGSLDPENRDLVLKFL
                130        140        150        160        170        180

           180         190         200
query   RALADKGATVVVATHSPLFRESADTIIKL
          .  .: ::...::.       ..     ::.:

pir:A9  LEMNREGKTVIIVTHDAYVAQQCHRIIELGEGK
                190        200        210
```

?

>>pir:D97884 [D97884] hypothetical protein ABC-NBD [impo (213 aa)
initn: 263 init1: 263 opt: 486 Z-score: 569.6 bits: 112.4 E(): 1e-23
Smith-Waterman score: 486; 39.713% identity (40.887% ungapped) in 209 aa overlap (1-203:1-209)

```
                10         20         30         40         50         60
query   MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQILV
        :::... :..: ... ..::..   : ..:..: ::::::::::: ::.:      ::.  .

pir:D9  MIELKNITKTIGGKVILDNLSLRIDQGDLVAIVGKSGSGKSTLLNLLGLIDGDYSGRYEI
                10         20         30         40         50         60

                70         80         90        100        110
query   EDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAVEKHKWPE-------IPQ
         :...  :..    .. ..::::.:::  :.::..::.::..    : :.         . .

pir:D9  FGQTNLAVNSAKSQTIIREHISYLFQNFALIDDETVEYNLMLALKYVKLPKKDKLKKVEE
                70         80         90        100        110        120

           120         130         140         150         160         170
query   VLHAVGLESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIEAL
        .:.  :::.   ..  : :::::::: :.::..::  ..::::::::.::   : .::.. :

pir:D9  ILERVGLSATLHQRVSELSGGEQQRIAVARAILKPSQLILADEPTGSLDPENRDLVLKFL
                130        140        150        160        170        180

           180         190         200
query   RALADKGATVVVATHSPLFRESADTIIKL
          .  .: ::...::.       ..     .:.:
```

putative
ABC Transporter.

```
pir:D9 LEMNREGKTVIIVTHDAYVAQQCHRVIELGEGK
              190       200       210

>>prf:2724351JF ABC transporter - Streptomyces avermitil   (248 aa)
 initn: 308 init1: 255 opt: 480  Z-score: 561.8  bits: 111.2 E(): 2.7e-23
Smith-Waterman score: 480;  40.845% identity (43.500% ungapped) in 213 aa overlap (2-203:9-219)

                      10             20        30        40
query         MIEINDLKKSFG-----VRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLG
              :.. .... .:      :  : : .:  :  ::.::. : :::::::::.. : .
prf:27 MGQMSNDAIQLRSVSRRYGAGGGAVTALDQ-VSLAFPRGTFTAVMGPSGSGKSTLLQCAA
               10        20        30         40        50

       50        60        70        80        90           100
query  TLDKPSSGQILVEDVDLLKLSTRKQRLYRKNTVGYLFQDYALIP----DRTVKFNLQLAV
        :.:.::.. : :..: ::: :  : :.. .:..:: . :.:    ... : . :.::
prf:27 GLDRPTSGSVTVGDTELTKLSETKLTLLRRDRIGFVFQAFNLLPSLTAEQNVALPLRLAG
      60        70        80        90       100       110

          110       120        130       140       150       160
query  EKHKWPEIPQVLHAVGL-ESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALD
       .. .   :. .::  :::.    ..:. :.:::.:::.::::.:.    : ....::::::
prf:27 RRPRKTEVREVLAQVGLGDRAGHRPT-EMSGGQQQRVALARALITRPDVLFGDEPTGALD
     120       130       140        150       160       170

            170       180        190       200
query  LTNSELVIEALRALAD-KGATVVVATHSPLFRESADTIIKL
        .:. :.  :.:...: . : :.:...::.:.     ::  .. :
prf:27 SQTSREVLTLLRGMVDSEGQTVIMVTHDPVAASYADRVVFLVDGRVNGELIGASAEDIAA
        180       190       200       210       220       230

prf:27 RMTKLEAAPC
        240

>>pir:B86626 [B86626] ABC transporter ATP-binding protei   (211 aa)
 initn: 283 init1: 283 opt: 474  Z-score: 555.8  bits: 109.8 E(): 5.9e-23
Smith-Waterman score: 474;  39.234% identity (40.394% ungapped) in 209 aa overlap (1-203:1-209)

               10        20        30        40        50        60
query  MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSGQILV
       ::::..: ::.  .:... :. ..  : :::. :.::.::::::: .: ..   ..:.
pir:B8 MIEIEELTKSYKGHIIFDKLNLRIPEGKMTAIYGTSGAGKSTLLNIIGLIEDYDDGKYYF
               10        20        30        40        50        60

               70        80        90       100             110
query  EDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAV------EKHKWPEIPQ
       .      ...     .:.: ..::::.. ::  :.:..  ::..:.      .:.:  .. .
pir:B8 NGQFAPPFNSSLALKMRRNKISYLFQNFALLEDETIEKNLEIALIYSRISKKEKRKKMKK
               70        80        90       100       110       120

          120       130       140       150       160       170
query  VLHAVGLESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVIEAL
       .:  ::..   .   :. :::::.::.:.::.:::.. ..::::::::.::  :. ::  :
pir:B8 LLLQVGINHRLNTKVYSLSGGEKQRVAIARALLKESQLILADEPTGSLDTENRNEVIALL
                130       140       150       160       170       180

          180       190       200
query  RALADKGATVVVATHSPLFRESADTIIKL
       :  .:::.::..::.  ..:.:.:..
pir:B8 RQEVDKGKAVVIVTHDSYLKEVSDLVIEIGE
               190       200       210

>>pir:D69433 [D69433] ABC transporter, ATP-binding prote   (226 aa)
 initn: 436 init1: 242 opt: 466  Z-score: 546.1  bits: 108.1 E(): 2e-22
Smith-Waterman score: 466;  39.583% identity (41.081% ungapped) in 192 aa overlap (19-203:25-216)
```

ABC transporter

ABC transporter

```
                      10        20        30        40        50
query          MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPS
                                     :.:.   :..:. : :::::::::: .: ::::.
pir:D6 MKVIELVDVYKIYRTAYYEVHALDGVSMEVEAGEFVAIMGPSGSGKSTLLNMIGCLDKPT
               10        20        30        40        50        60

          60        70        80        90       100
query  SGQILVEDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQL------AVEKHK
       .:..... :     :: :.     :....:..::.: :::  :.   :..:      ... ..
pir:D6 KGEVIINGVKTSGLSDRELTKLRRDSIGFIFQQYNLIPTLTALENVELPMIFRGVARAER
               70        80        90       100       110       120

      110       120       130       140       150       160
query  WPEIPQVLHAVGLESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSE
        .  ..:..::.: .  ..      :.:::.:::.:.::.: .::.:.: :::::  ::  ...
pir:D6 ERRAKELLKVVGIEELADRRPREMSGGQQQRVAIARALANNPKILLCDEPTGNLDTKSGR
              130       140       150       160       170       180

      170       180       190       200
query  LVIEALRALADK-GATVVVATHSPLFRESADTIIKL
        :.  :. : .. :.:::..::.: .  : :: .:..
pir:D6 QVMGILKNLNEENGVTVVLVTHDPSLSEYADRVIRIRDGKVVEDVY
              190       200       210       220
```

>>sp:Y065_MYCPN [P75612] Hypothetical ABC transporter AT (465 aa)
initn: 286 init1: 243 opt: 466 Z-score: 542.2 bits: 108.4 E0 : 3.3e-22
Smith-Waterman score: 466; 36.667% identity (38.308% ungapped) in 210 aa overlap (1-201:231-440)

```
                                                10        20
query                                MIEINDLKKSF--GVRI--LWQGLSHKFLP
                                     .::....  :.   ::   . .:.. :.
sp:Y06 HLFLKNEVKKVTWLNEPRAKKESVTPDEEHIIELKNVYKYITNGVTTNAVLKGIDLKLKA
              210       220       230       240       250       260

          30        40        50        60        70        80
query  GTMTALTGASGSGKSTLLNCLGTLDKPSSGQILVEDVDLLKLSTRKQRLYRKNTVGYLFQ
        . .. : :::::.:::: .. .:.:::::...:.   ... ..  :.   .:.: :::.::
sp:Y06 HDFIVILGPSGSGKTTLLNIISGMDRPSSGSVVVNGQEMICMNDRQLTNFRRNYVGYIFQ
              270       280       290       300       310       320

          90       100       110             120       130       140
query  DYALIPDRTVKFNLQLAVEKHKWPE-----IPQVLHAVGLESFEEKPVFELSGGEQQRTAL
       .:.:.:  ::. :...... .. :.      : ..:.::.:.. ...:   ::::.:::...
sp:Y06 QYGLLPNLTVRENVEVGANLQRNPDKRINIDELLEAVGMKHLQKKLPNELSGGQQQRVSI
              330       340       350       360       370       380

         150       160       170       180       190       200
query  ARVLLKNPRIILADEPTGALDLTNSELVIEALRALADK-GATVVVATHSPLFRESADTII
       ::.. ::: .:..:::::::::  ...:.. . :. ..  .:.:..::. :. . ::.:
sp:Y06 ARAFAKNPLLIFGDEPTGALDLEMTQIVLKQFLAIKQRYKTTMVIVTHNNLIAQLADLVI
              390       400       410       420       430       440

query  KL

sp:Y06 YVADGKIQALQANPNPKQVEDINWI
              450       460
```

ABC transporter

>>tr:Q9L0J9 [Q9L0J9] Putative ABC-transporter ATP-bindin (246 aa)
initn: 311 init1: 233 opt: 463 Z-score: 542.2 bits: 107.5 E0 : 3.3e-22
Smith-Waterman score: 463; 44.262% identity (46.023% ungapped) in 183 aa overlap (27-203:36-217)

```
                 10        20        30        40        50
query     MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKPSSG
```

```
                                          :. ::.  : ::::::::::. : .  ::. :. ::
tr:Q9L AIRLSSVSRRYGAGEGTVTALDDVSLALRRGSFTAVMGPSGSGKSTLLQCAAGLDRPTSG
          10         20         30         40         50         60

          60         70         80         90          100        110
query  QILVEDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRT----VKFNLQLAVEKHKWPEI
       ...:  ..:  :: :.  : :.. ::. :: . :. :.  :    : .  :. :: ..     ..
tr:Q9L SVVVGGTELTGLSQRRLTLLRRERVGFVFQAFNLLPSLTAAQNVALPLRLAGRRPPRGRV
          70         80         90        100        110        120

           120        130        140        150        160        170
query  PQVLHAVGL-ESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNSELVI
       ..:. ::: .  ... :. :::::. ::: :::: :.   :.... ::::::::::  ...  :.
tr:Q9L REALRQVGLADRARHRPA-ELSGGQQQRVALARALITRPQVLFADEPTGALDSRTGREVL
          130        140        150        160        170        180

             180        190        200
query  EALRALAD-KGATVVVATHSPLFRESADTIIKL
       :::. :: . : ::::. ::. :.    :: ..  :
tr:Q9L TLLRAMADGEGRTVVMVTHDPVAASYADRVLFLVDGRVHDELTGSGPDGIATRMTRLEAA
          190        200        210        220        230        240

tr:Q9L PC


>>pir:B69377 [B69377] ABC transporter, ATP-binding prote  (228 aa)
 initn: 483 init1: 234 opt: 461  Z-score: 540.3  bits: 107.1 E(): 4.3e-22
Smith-Waterman score: 461;  39.000% identity (40.625% ungapped) in 200 aa overlap (11-203:18-216)

                    10         20         30         40         50
query          MIEINDLKKSFGVRILWQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLDKP
                        . :: : . :. :     : .... : :::::::::: . : ::::
pir:B6 MKVVELRNVYKIYRTEYYEVRAL-DGVSMDVEEGEFVVIMGPSGSGKSTLLNLIGCLDKP
                10         20         30         40         50

            60         70         80         90         100
query  SSGQILVEDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAV------EKH
       . :. :.  :.  . :.  .    :. :. :. :: : :::  :.  :. : .        ...
pir:B6 TEGEVLINGVETSSLNDNRLTELRRDTIGFIFQTYNLIPTLTALENVELPMIFKGVGRRE
       60         70         80         90         100        110

       110        120        130        140        150        160
query  KWPEIPQVLHAVGLESFEEKPVFELSGGEQQRTALARVLLKNPRIILADEPTGALDLTNS
       . .  .. :.  :::::  ..    :. :::. :::. :. :: : . :: :. : ::::: :: ..
pir:B6 REERAKELLKNVGLEKEMNRKPNEMSGGQQQRVAIARALANNPKILLCDEPTGNLDTKSG
     120        130        140        150        160        170

       170        180        190        200
query  ELVIEALRALADK-GATVVVATHSPLFRESADTIIKL
       : :. : :   .   :. :. .. :: : . . . : .. :
pir:B6 EQVMEIIRHQNEVLGVTVILVTHDPSLAKYGDRVIRLRDGKIESVENVS
     180        190        200        210        220

>>prf:2719186AYG ABC transporter - Sulfolobus tokodaii>t  (232 aa)
 initn: 415 init1: 240 opt: 460  Z-score: 539.1  bits: 106.8 E(): 5e-22
Smith-Waterman score: 460;  35.681% identity (37.811% ungapped) in 213 aa overlap (1-201:6-218)

                   10         20         30         40         50
query          MIEINDLKKSFGVRIL----WQGLSHKFLPGTMTALTGASGSGKSTLLNCLGTLD
                .: :. ::: . .. .     .:.. :.  : . ... : :::::.::. .: ::
prf:27 MSEDELIIIENLKKIYKIKNVEFPALRGINLKIYKGEFLGIAGPSGSGKTTLLDMIGLLD
                10         20         30         40         50         60

            60         70         80         90         100        110
query  KPSSGQILVEDVDLLKLSTRKQRLYRKNTVGYLFQDYALIPDRTVKFNLQLAVEKHKWP-
```

Arch Microbiol (2003) 180:88–100
DOI 10.1007/s00203-003-0561-4

The Reference 2

ORIGINAL PAPER

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A transporter of *Escherichia coli* specific for L- and D-methionine is the prototype for a new family within the ABC superfamily

Received: 17 March 2003 / Revised: 5 May 2003 / Accepted: 7 May 2003 / Published online: 19 June 2003

Abstract An ABC-type transporter in *Escherichia coli* that transports both L- and D-methionine, but not other natural amino acids, was identified. This system is the first functionally characterized member of a novel family of bacterial permeases within the ABC superfamily. This family was designated the methionine uptake transporter (MUT) family (TC #3.A.1.23). The proteins that comprise the transporters of this family were analyzed phylogenetically, revealing the probable existence of several sequence-divergent primordial paralogues, no more than two of which have been transmitted to any currently sequenced organism. In addition, MetJ, the pleiotropic methionine repressor protein, was shown to negatively control expression of the operon encoding the ABC-type methionine uptake system. The identification of MetJ binding sites (in gram-negative bacteria) or S-boxes (in gram-positive bacteria) in the promoter regions of several MUT transporter-encoding operons suggests that many MUT family members transport organic sulfur compounds.
Electronic Supplementary Material Supplementary material is available for this article if you access the article at http://dx.doi.org/10.1007/s00203-003-0561-4. A link in the frame on the left on that page takes you directly to the supplementary material.

Keywords Transport · Methionine · MetD · ATP-binding cassette · *E. coli*

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Introduction

Methionine transport and its regulation have been extensively studied in both *Escherichia coli* and the phylogenetically related bacterium *Salmonella typhimurium*. *E. coli* has been shown to have two transport systems for L-methionine (Kadner 1974) but only one system for D-methionine (Kadner 1977). Spontaneous mutants selected for their capacity to grow on toxic methionine analogues were generated. A *metD* mutant lacks both high-affinity uptake activity for L-methionine (Kadner 1974) and lower affinity uptake activity for D-methionine (Kadner 1977; Kadner and Watson 1974). Specificity of the transport system for L- and D-methionine and related compounds has been examined (Kadner 1974, 1977), but inhibitory studies with other amino acids have not been reported. A *metD* mutant has also been isolated in *S. typhimurium* and exhibits characteristics similar to those of the corresponding mutant in *E. coli* (Ayling and Bridgeland 1972; Ayling et al. 1979; Betteridge and Ayling 1975; Poland and Ayling 1984).

Studies of energy coupling for methionine uptake in *E. coli* have suggested that transport is driven by phosphate bond energy, presumably ATP (Kadner and Winkler 1975). Moreover, the MetD transport system has been shown to be sensitive to osmotic shock and to inhibition by arsenate in both *E. coli* and *S. typhimurium* (Cottam and Ayling 1989; Kadner and Winkler 1975). It was therefore suggested that the major uptake system is an ATP-binding cassette (ABC) transporter. ABC transporters usually consist of a transmembrane protein, a cytoplasmic ATP-hydrolyzing (ABC) protein, and at least one substrate-binding receptor.

The *metD* mutation was mapped to 4.8 minutes on the *E. coli* chromosome (Berlyn 1998). Mapping of the *metD*

locus in *S. typhimurium* has revealed that several genes, when mutated, give rise to the *metD* phenotype (Grundy and Ayling 1992). In the work reported here, we identify an ABC transporter mapping very near 4.8 minutes, which proves to be the MetD transporter.

Methionine biosynthetic genes in *E. coli* are regulated by the MetJ repressor (Greene 1996; Sekowska et al. 2000; Weissbach and Brot 1991), and a MetJ DNA binding site consensus sequence has been derived (Saint-Girons et al. 1984). Evidence is available suggesting that methionine transport is also regulated by MetJ since cells grown in medium containing methionine have lower levels of methionine transport (Kadner 1975), and methionine auxotrophs with a *metJ* mutation have higher transport activity (Kadner 1975, 1977). The recent identification of a MetJ binding site in the promoter region of the ABC transporter mentioned above (Liu et al. 2001) corroborated this hypothesis.

Recently, Gal et al. (2002) and Merlin et al. (2002) reported growth studies that led to the tentative molecular identification of this transporter (*abc-yaeE-yae-C*) which they renamed *metNIQ*. The MetJ protein, in the presence of methionine, but not in its absence, was also shown to repress expression of the operon. Although no transport studies have been reported, it has been suggested that the three-gene cluster encodes the methionine transporter characterized physiologically by Kadner and his collaborators.

In this study, we confirm and substantially extend the previously reported work. We (1) report the first transport studies with this system, revealing that MetD transports both L- and D-methionine and probably formyl methionine; (2) resolve the question as to whether both L- and D-methionine are recognized by the same receptor; (3) provide evidence that NlpA (Yamaguchi and Inouye 1988; Yu et al. 1986) may, under certain conditions, serve as a poor L- and D-methionine receptor, feeding inefficiently into MetD; (4) show that MetJ represses expression of the *metD* operon; and (5) provide detailed phylogenetic data that define a novel family within the ABC superfamily, which we have called the methionine uptake transporter (MUT) family (TC #3.A.1.23).

Materials and methods

Bacterial strains and media

E. coli strains and plasmids used in this study are listed in Table 1. All studies were conducted in the genetic background of strain BW25113. Bacteria were cultured in Luria-Bertani (LB) broth or M9 minimal medium at 37 °C (Sambrook et al. 1989). When appropriate, ampicillin (Ap) and/or kanamycin (Km) was/were added to the medium at 100 and/or 25 μg/ml, respectively. Unless otherwise stated, chemicals were purchased from Sigma-Aldrich.

Generation of deletion mutants

Deletion mutants were generated using the methods described by Datsenko and Wanner (2000). To prepare competent cells for transformation, BW25113 containing pKD46 was cultured at 30 °C in SOB broth (Sambrook et al. 1989) containing ampicillin and 2 mM L-arabinose. When the OD_{600} reached 0.5, the culture was centrifuged at 2,000×*g* for 5 min, and the cells were washed three times with cold 10% glycerol before being resuspended in a minimal volume of 10% glycerol (1% of the original culture). The competent cells were stored at −80 °C prior to use. PCR methods were used to clone the kanamycin gene from pKD4 using the primers described in Table 2. The PCR products were purified using a Qiagen kit, treated with *Dpn*I, and repurified by electrophoresis. BW25113 competent cells were transformed with the kanamycin gene by electroporation (Gene Pulser, pulse controller at 200 Ω, capacitance at 250 μFD, and voltage at 2.5 kV). After electroporation, the cells were grown with shaking in 1 ml of SOC (Sambrook et al. 1989) at 37 °C for 1 h, and the cultures were plated onto LB agar medium containing kanamycin. The kanamycin-resistant transformants were purified on new kanamycin-LB plates. The mutants in which the target genes were replaced by the kanamycin gene were verified by three PCRs using bacterial DNA as the template. The first PCR used forward primer k_2(5′-C G G T G C C C T G A A T G A A C T G C-3′) and reverse primer k_1 (5′-C G G C C A C A G T C G A T G A A T C C-3′) (Datsenko and Wanner 2000), both of

Table 1 Strains and plasmids used in this study

Strain/plasmid	Genotype	Reference
Strain		
LJ 3001 (BW25113)	*lacI*^{q}*rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner 2000
LJ 3015	BW25113 Δ*abc-yaeE* (Δ*metNI*)	This study
LJ 3016	BW25113 Δ*yaeC* (Δ*metQ*)	This study
LJ 3017	BW25113 Δ*nlpA*	This study
LJ 3018	BW25113 Δ*yaeC* Δ*nlpA*	This study
LJ 3019	BW25113 Δ*yaeE-abc* Δ*ykfD* (Δ*metNI* Δ*nmuP*)	This study
LJ3020	BW25113 Δ*ykfD*	This study
LJ 3021	BW25113 Δ*metJ*	This study
Plasmid		
pKD46	*oriR101 repA101*(ts) *araBp-gam-bet-exo* Ap^{r}	Datsenko and Wanner 2000
pKD4	*oriR*$_{γ}$ Ap^{r} Km^{r}	Datsenko and Wanner 2000
pCP20	λ*cI857*(ts) ts-rep	Datsenko and Wanner 2000
pBAD24	Expression vector, Ap^{r}	Guzman et al. 1995
pBAD24-metD	pBAD24 carrying *metD* operon	This study
pBAD24-metJ	pBAD24 carrying *metJ*	This study

Table 2 Primers used for generation and verification of BW25113 mutants. For all gene mutations, pKD4 was used as the template for cloning the kanamycin gene

Gene	Primers (5′→3′)
abc-yaeE (*metNI*)	Generation
	GATGCGGTCGCCTGCGAACTGAATTAAATAAACCAGAATGACCAGGTGTAGGCTGGAGCTGCTTC (forward)
	CTTAATGACGATATAAATAATCAATGATAAAACTTTCGAATATCCATATGAATATCCTCCTTAG (reverse)
	Verification
	abc-yaeE 3: CGTTACTTGCGAGTGACAGC
	abc-yaeE 4: GCATGTGACGCTAGTATCGC
yaeC (*metQ*)	Generation
	TTACAAATTGTGGAAACAGCCTAAAAATTACCAGCCTTTAACAGCGTGTAGGCTGGAGCTGCTTC (forward)
	AAGGAATAAGGTATGGCGTTCAAATTCAAAACCTTTGCGGCAGTGCATATGAATATCCTCCTTAG (reverse)
	Verification
	yaeC 3: ACAGCCGCTTAGCATGAGTG
	yaeC 4: AATTCAGTTCGCAGGCGACC
nlpA	Generation
	ACCGCAGCGACCTTACCGCTATAGTCAGGTAATCATTAATAAAAGGTGTAGGCTGGAGCTGCTTC (forward)
	TGAGAATTACCAGCCAGGCACCGCGCCACCGTTAAAAATGGTTTCCATATGAATATCCTCCTTAG (reverse)
	Verification
	nlpA 3: CGTGGTCAGTAAGAAGTGCC
	nlpA 4: GCTGCTGATTCTGTCATCGG
ykfD (*mmuP*)	Generation
	GGTTGACTTTGCATTCTGTTAACAAACGCGGTATAACAAACCGTGTAGGCTGGAGCTGCTTC (forward)
	GGTTGAGTAAGGAAATAAGCACCATAGCACAACGCAACAAACCATATGAATATCCTCCTTAG (reverse)
	Verification
	ykfD 3: GACTTGTTCGCACCTTCC
	ykfD 4: GGCTGTCGGCTAAGTTAC
metJ	Generation
	TGGTCTGGTCTCAATTTATTGACGAAGAGGATTAAGTATCTCATGGTGTAGGCTGGAGCTGCTTC (forward)
	TAGCGCATCAGGCGATTCCACTCCGCGCCGCTCTTTTTTGCTTTACATATGAATATCCTCC AATG (reverse)
	Verification
	metJ 3: CAACTGTGTGGTCTGGTCTC
	metJ 4: TGCGATGAGCGAGAGATCTG

which are designed from the internal kanamycin gene. The second PCR used primer k_2 and primer 4 (reverse, Table 2). The third PCR used primers 3 and 4 (Table 2). To delete the kanamycin gene from the chromosome, pKD46 was removed from the cells by growing the bacteria at 37 °C, and then pCP20 was introduced by transformation. The transformants containing pCP20 were grown overnight with shaking at 42 °C, and the cultures were plated on LB agar without antibiotics. Colonies were tested for sensitivity to kanamycin and ampicillin. To verify the loss of the kanamycin gene, the last PCR using primers 3 and 4 (see above, Table 2) was repeated. Growth experiments were carried out in M9 minimal medium, with $MgCl_2$ replacing $MgSO_4$ and with 20 μM L-methionine or 100 μM D-methionine serving as the sole sulfur source.

DNA manipulations and gene cloning

Standard methods were used for chromosomal DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (Sambrook et al. 1989). Plasmids were isolated using spin miniprep kits (Qiagen, Chatsworth, Calif., USA), and PCR products were purified using Qiaquick purification kits (Qiagen). For gene cloning, the *metD* operon (*abc-yaeE-yaeC*) and *metJ* gene were amplified from wild-type *E. coli* BW25113 chromosomal DNA by PCR. The following primers were used for gene amplification (restriction sites *Kpn*I, *Xba*I and *Hind*III are underlined) – for *metD*, 5′-CGC<u>GGTACC</u>GATAAAACTTTCG AATATCACC-3′ and 5′-CGCC<u>TCTAGA</u>TTACA AATTGTGGAAACAGCC-3′, and for *metJ*, 5′-GAA <u>TCTAGA</u>CATGGCTGAATGGAGCGGCG-3′ and 5′-CGCC<u>AAGCTT</u>AGTATTCCCACGTCT CCG-3′. The PCR products were purified, treated with *Kpn*I and *Xba*I (for *metD*) or with *Xba*I and *Hind*III (for *metJ*), and then cloned into pBAD24 (Guzman et al. 1995).

Transport assays

Cells grown in M9 minimal medium were harvested in the exponential growth phase, washed once in Tris-maleate (TM) buffer, pH 7.0, and resuspended in the same buffer containing 0.5% D,L-lactate plus 100 μg chloramphenicol/ml. Uptake studies were conducted at 37 °C over a 10-min time interval with the cell density at an OD_{600} of 0.10 and 0.40 and the methionine concentration at 0.5 μM (50,000 cpm/ml; 55 μCi/μmol) and 5.0 μM (50,000 cpm/ml; 5.5 μCi/μmol), respectively, for the L- and D-isomers. 1-[^{14}C]L- and D-methionine were purchased from American Radiolabeled Chemicals. Aliquots (100 μl) were periodically removed from the 1-ml cell suspensions. Cells were transferred to 10 ml of ice-cold TM buffer, filtered (0.45-μm Millipore filters) and washed twice with the same buffer. After drying the filters, radioactivity was measured by scintillation counting using 10 ml of Biosafe NA scintillation fluid (Research Products International, Mt. Prospect, Ill., USA). For comparison of the uptake of L-methionine between

the *abc-yaeE* and the *abc-yaeE mmuP* mutants, L-methionine concentrations of 13 and 103 μM (12 μCi/μmol) were used since the low-affinity L-methionine transporter was analyzed. Initial uptake rates were inhibited over a 5-min time interval for both L- and D-methionine. Unless otherwise stated, the concentration of the non-radioactive inhibitory amino acid was ten times the methionine concentration. The uptake activity attributed to MetD alone was obtained by subtracting the activity remaining in the absence of MetD function from the wild-type activity (e.g., see Table 3), assuming that loss of MetD does not activate some other transporter.

Computer methods

Sequences of the proteins that comprise the three constituents of MUT family permeases were obtained by initial BLAST searches (Altschul et al. 1997) using the sequences of the three *E. coli* MetD permease constituents as query. The resulting hits were filtered through a program manipulating the BLAST program to eliminate the sequences more related to other families in the ABC superfamily (C. Tran and M.H. Saier, Jr., unpublished program).

Multiple sequence alignments were constructed using the ClustalX program (Thompson et al. 1997). The gap penalty and gap extension values used with the ClustalX program were 10 and 0.1, respectively, although other combinations were tried. The HMMTOP (Tusnady and Simon 1998, 2001) and TMHMM (Krogh et al. 2001; Sonnhammer et al. 1998) programs were used to determine the predicted numbers of transmembrane segments. Phylogenetic trees were derived from alignments generated with the ClustalX program using the BLOSUM 62 scoring matrix. The trees were drawn using the TreeView program (Page 1996). Complementary trees were constructed using the Phylo_win program (Galtier et al. 1996) with the neighbor-joining method and PAM distances as the model of evolution. This study was conducted independently for the three protein constituents of the ABC transporters that comprise the MUT family, and the sequences obtained were checked manually to see whether all three ABC elements had been identified for each transporter.

G+C content was analyzed with the GeeCee program (Rice et al. 2000), and codon usage was analyzed with the Countcodon program from the Codon Usage Database website (http://www.kazusa.or.jp/codon/countcodon.html). The lipoproteic structure of the receptors was predicted with the Lipop section of the PSORT program (http://psort.nibb.ac.jp). S-boxes were predicted with the RNApattern program (Vitreschak et al., unpublished program).

Results

Growth studies

Figure 1 shows the growth of the isogenic strains described above with 100 μM D-methionine as the sole source of sulfur. The wild-type strain, the *nlpA* mutant, and the *ykfD* (Thanbichler et al. 1999) mutant grew equally well, but the *abc-yaeE* mutant and the *yaeC nlpA* double mutant as well as the *abc-yaeE-ykfD* triple mutant grew very poorly. Both the growth rates and growth yields were substantially depressed. These effects were completely reversed by inclusion of a plasmid (pBAD24) bearing the *metD* (*abc-yaeE-yaeC*) operon (data not shown, see supplementary Fig. S1 in the electronic supplementary material). The *yaeC* single mutant grew substantially better than the two double mutants but much less well than the wild-type strain. When 20 μM L-methionine served as the sole source of sulfur, the difference between the wild-type and the double mutants was less pronounced than when D-methionine

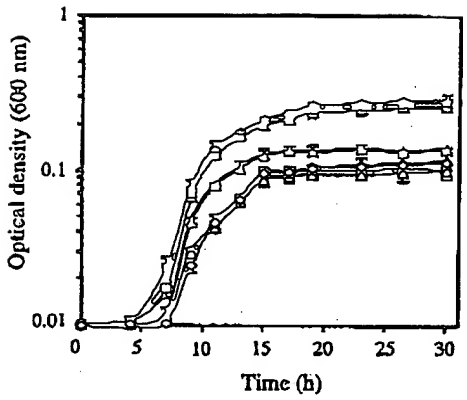


Fig. 1 Growth of *Escherichia coli* as a function of time in M9 minimal medium in which $MgSO_4$ was replaced by 1 mM $MgCl_2$ and D-methionine was added at a concentration of 100 μM as the sole sulfur source present. The growth experiments were conducted three times, and the results were averaged. The following strains were examined: wild-type (BW25113) (◇), *Δabc-yaeE* (□), *ΔyaeC* (△), *ΔnlpA* (○), *ΔyaeC-ΔnlpA* (◇), *ΔykfD* (□) and *Δabc-yaeE ΔykfD* (△). *Error bars* indicate standard deviations

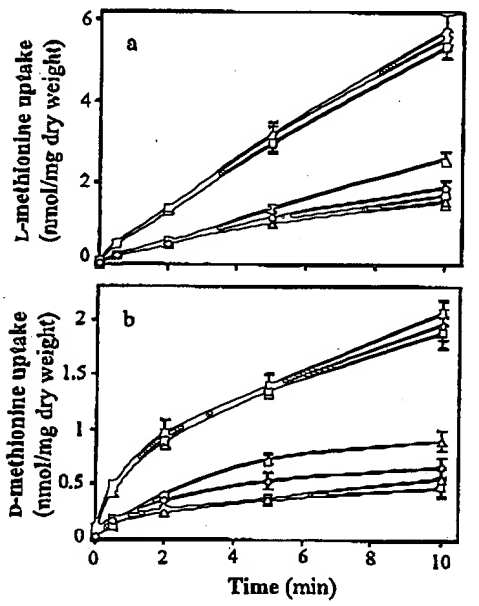


Fig. 2 Uptake of 1-[^{14}C]L-methionine (a) and 1-[^{14}C]D-methionine (b) by wild-type (BW25113) (◇), *Δabc-yaeE* (□), *ΔyaeC* (△), *ΔnlpA* (○), *ΔyaeC-ΔnlpA* (◇), *ΔykfD* (□) and *Δabc-yaeE ΔykfD* (△) cells. Cells grown in M9 minimal medium were prepared as described in Materials and methods. Uptake assays were carried out at 37 °C over a 10-min time interval with the optical densities (600 nm) at 0.1 (a) or 0.4 (b) and the methionine concentrations at 0.5 μM (55 μCi/μmol) or 5 μM (5.5 μCi/μmol) (both at 50,000 cpm/ml), respectively, for the L- or D-isomers. Values are expressed in pmol L- or D-methionine retained per mg bacterial dry weight. The experiment was conducted three times, and the results reported represent an average of the values obtained. *Error bars* indicate standard deviations

was used (Fig. 1; data not shown). However, depressed growth suggested that the transporter could accept both D- and L-methionine as substrates.

Transport studies

Figure 2 shows the uptake of [^{14}C]L-methionine (Fig. 2a, 0.5 µM) and [^{14}C]D-methionine (Fig. 2b, 5 µM), respectively. Relative rates of uptake of the two substrates by the wild-type and mutant strains were essentially the same. Thus, the wild-type, and the mutant strains *ykfD* and *nlpA* took up the amino acids at nearly the same rate; the two double mutants (*abc-yaeE* and *yaeC nlpA*) took up both substrates poorly, and the *yaeC* single mutant took up both substrates poorly but slightly better than the double mutants. These depressed uptake rates were largely reversed by inclusion of the plasmid-encoded *metD* operon in these strains (data not shown; see Fig. S2 in the electronic supplementary material). It was therefore concluded that: (1) both L- and D-methionine are substrates of the Abc-YaeE-YaeC transporter, and (2) the binding receptor YaeC, and possibly NlpA, can activate both D- and L-methionine uptake. If NlpA acts as a receptor for the Abc-YaeE system, it is a much less effective receptor than YaeC. For both isomers, residual uptake was observed in the *abc-yaeE* mutant, suggesting that a second transporter exists both for L- and for D-methionine. We tested the transport of L- and D-methionine at concentrations of 10 and 100 µM in the *mmuP abc-yaeE* triple mutant, but no significant difference was observed relative to the *abc-yaeE* mutant (data not shown). This result is consistent with the indistinguishable growth rates between the *abc-yaeE-yaeC* and the *abc-yaeE-yaeC mmuP* mutants observed by Gal et al. (2002) in a methionine auxotrophic strain grown in minimal medium supplemented with L-methionine. It can therefore be assumed that the S-methylmethionine permease MmuP is not responsible for the residual transport of L- or D-methionine observed in the *abc-yaeE* mutant.

Inhibition studies

Table 3 summarizes the inhibitory effects of several non-radioactive amino acids present at ten-fold the concentration of the radioactive amino acid on uptake of both L- and D-methionine. Uptake of L-methionine by the wild-type bacteria was strongly inhibited by L-methionine and weakly

Table 3 Inhibition of L-methionine (top) and D-methionine (bottom) uptake by the L- and D-isomers of several amino acids. Assays were done as described in Materials and methods with the non-radioactive inhibitory amino acids at 10× the concentrations of the radioactive substrate. Rate of uptake is expressed in pmol/(min×mg dry weight). Wild type–*Δabc-yaeE* is the uptake activity attributed to MetD alone

Inhibitor	Wild-type		*Δabc-yaeE*		Wild-type–*Δabc-yaeE*	
	Rate	%	Rate	%	Rate	%
–	688.8±88	100	230.4±16	100	458.5±72	100
L-Methionine	47.5±5	7	41.8±8	18	5.7±3	1
D-Methionine	635.3±45	92	298.6±16	129	336.8±30	73
N-Formyl-L-methionine	268.3±18	39	258.9±13	112	9.3±5	2
L-Alanine	627.4±56	91	176.9±20	77	451.1±35	98
D-Alanine	649.5±40	94	281.6±22	122	367.9±18	80
L-Leucine	581.8±40	84	179.7±16	78	402.1±24	88
D-Leucine	677.5±56	98	245.6±36	107	431.9±20	94
L-Valine	528.1±50	77	159.1±18	69	369.0±31	80
L-Serine	833.2±105	121	271.7±17	118	561.5±88	122
D-Serine	590.3±57	86	304.8±25	132	285.5±34	62
L-Threonine	612.7±76	89	151.7±25	66	461.0±51	101
D-Threonine	649.8±72	94	245.6±34	107	404.1±38	88
Inhibitor	Wild-type		*Δabc-yaeE*		Wild-type–*Δabc-yaeE*	
	Rate	%	Rate	%	Rate	%
–	287.8±31	100	68.4±11	100	219.3±20	100
D-Methionine	34.2±4	12	28.8±5	42	5.4±1	2
L-Methionine	66.7±12	23	36.5±5	53	31.1±6	14
N-Formyl-L-methionine	71.6±11	25	45.8±8	67	25.73±3	12
L-Alanine	273.0±25	95	77.8±9	114	195.2±16	89
D-Alanine	306.5±29	106	73.1±7	107	233.33±22	106
L-leucine	269.1±24	94	75.4±14	110	193.7±10	88
D-Leucine	258.2±26	90	84.0±4	123	174.2±22	79
L-Valine	232.6±21	81	65.3±9	95	167.2±12	76
L-Serine	327.5±36	114	70.8±10	103	256.7±26	117
D-Serine	338.3±23	118	85.6±7	125	252.8±17	115
L-Threonine	345.3±33	120	84.0±11	123	261.3±22	119
D-Threonine	329.8±33	115	87.9±6	128	24L9±27	110

inhibited by *N*-formyl L-methionine. However, no other amino acid inhibited strongly. Uptake by the *abc-yaeE* mutant was most strongly inhibited by L-methionine and to a lesser extent by L-threonine, L-valine, L-alanine, and L-leucine. When uptake of D-methionine was studied in wild-type *E. coli*, L-methionine was most inhibitory followed by *N*-formyl L-methionine and D-methionine in that order, but no other amino acid inhibited. In the *abc-yaeE* mutant, only L-methionine inhibited strongly. The mild increase of transport rate observed in the presence of some amino acids is unexplained. The results are consistent with the conclusion that the MetD transporter is specific for L- and D-methionine as well as *N*-formyl-L-methionine. The relative inhibitory effects of L- and D-methionine on uptake of these two radioactive substrates are in line with the relative affinities reported by Kadner (1974, 1977) (K_m values of 75 nM for L-methionine (Kadner 1974) and 1.2 µM for D-methionine (Kadner 1977)). Inhibition studies of D-methionine uptake were also conducted with 100- and 1,000 fold excess of both L- and D-methionine. The results were consistent with the existence of a second low-affinity transporter for methionine (data not shown). Surprisingly, the uncharacterized methionine transporter present in the mutant is also fairly specific for methionine.

The effects of energy poisons were also examined (data not shown; see Fig. S3 in the electronic supplementary material). The conditions used were essentially the same as those reported in Zhang et al. (2003). Five mM sodium arsenate virtually abolished uptake of both L- and D-methionine under the same conditions used in the experiment reported in Fig. 2. FCCP (carbonyl cyanide 4-trifluoromethoxyphenylhydrazone) at a concentration of 2 µM was substantially less inhibitory (see Fig. S3 in the electronic supplementary material). The results clearly suggest that uptake is energy dependent and are consistent with the expectation that ATP is the energy source.

Regulation of *metD* operon expression by MetJ

A *metJ* knockout mutant was constructed, and *metJ* was cloned into plasmid pBAD24 for complementation studies. Using [^{14}C]D-methionine as the uptake substrate, the effects of *metJ* expression plus and minus L- and D-methionine were studied (Fig. 3). In Fig. 3a, it can be seen that the presence of either L- or D-methionine substantially reduced uptake of D-methionine into wild-type cells. In Fig. 3b, the same experiment conducted with the *metJ* mutant revealed that (1) the loss of *metJ* enhanced uptake above that observed for the wild-type strain grown without methionine and (2) methionine present during growth did not exert a repressive effect. In Fig. 3c, it can be seen that the plasmid bearing *metJ* depressed methionine uptake activity in the wild-type background and depressed the much greater activity of the *metJ* mutant even more. As expected, the wild-type strain expressing *metJ* on the plasmid exhibited lower D-methionine uptake than observed for the *metJ* mutant bearing the same plasmid. These

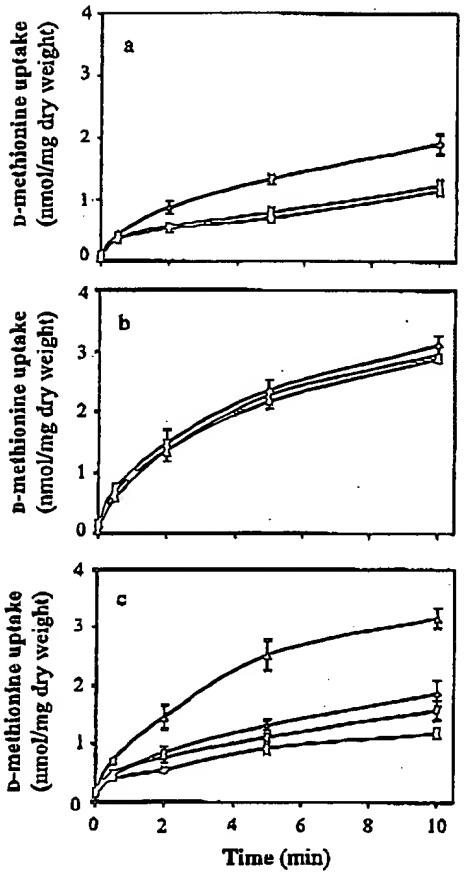


Fig. 3a–c Uptake of [^{14}C]D-methionine by cells grown in M9 medium with or without L- or D-methionine. Uptake experiments were conducted as described in the legend to Fig. 2. a Wild-type cells grown without methionine (◇), with 25 µM L-methionine (□), or with 25 µM D-methionine (△). b The same conditions and symbols were used for the *metJ* mutant. c Complementation studies with the *metJ* bearing pBAD24 plasmid. Wild-type cells bearing pBAD24 (◇), wild-type cells bearing pBAD24-*metJ* (□), *metJ* mutant with pBAD24 (△), *metJ* mutant with pBAD24-*metJ* (○). All cells in (c) were grown in medium M9 containing 100 µg ampicillin ml^{-1} and 2 mM L-arabinose

results are in accord with the expected *metJ* gene dosages. Measurements of L-methionine uptake were similar, but differences were substantially less pronounced as expected (data not shown).

Phylogenetic studies

Our preliminary results suggested that the *E. coli* ABC methionine transporter identified by Gal et al. (2002) and Merlin et al. (2002) and in the work described here belongs to a novel family within the ABC superfamily. Surprisingly, this family is more closely related to the polar amino acid uptake transporter (PAAT) family (TC# 3.A.1.3) than to the hydrophobic amino acid uptake transporter (HAAT)

Table 4 Members of the methionine uptake transporter (MUT) family. All full-length homologues of the membrane protein were predicted to exhibit 5 TMSs using the TMHMM or HMMTOP programs. * indicates an organism for which the complete genome sequence is not available in GenBank. GenBank accession numbers are given. Lipoprotein: *Y*, yes, the receptor is predicted to be a lipoprotein; *N*, no, it is not predicted to be a lipoprotein; *?*, the prediction is uncertain

Phylogenetic cluster	Organism	Source	Protein abbreviation	ABC proteins		Membrane proteins		Substrate-binding receptors		
				Accession number	Size (aa)	Accession number	Size (aa)	Accession number	Size (aa)	Lipoprotein ?
1	*Salmonella typhimurium*	γ-Proteobacteria	Styl	AAL19210	343	AAL19209	217	AALI9208	271	Y
	Escherichia coli	γ-Proteobacteria	Eco	AAC73310	343	AAC73309	217	AAC73308	271	Y
				–		–		AAC76685	272	Y
	Yersinia pestis	γ-Proteobacteria	Ypel	CAC89916	343	CAC89915	217	CAC89914	271	Y
	Vibrio cholerae	γ-Proteobacteria	Vch	AAF94069	344	AAF94068	225	AAP94067	275	Y
	Pasteurella multocida	γ-Proteobacteria	Pmu	AAK03812	344	AAK03813	229	AAK03814	276	Y
	Haemophilus influenzae	γ-Proteobacteria	Hin	AAC22280	345	P46492	198	AAC22279	273	Y
2	*Lactococcus lactis*	Firmicutes; Bacillales	Lla	CAB59828	368	AAK04421	231	CAB59827	286	N
	Streptococcus pneumoniae	Firmicutes; Bacillales	Spn	AAK98953	353	AAK98954	230	AAK98951	284	Y
	Streptococcus pyogenes	Firmicutes; Bacillales	Spy	AAM7884I	354	AAM78842	230	AAM78840	281	Y
	Streptococcus mutans	Firmicutes; Bacillales	Smu	AAL04079	354	AAL04080	229	AAL04077	280	Y
3	*Fusobacterium nucleatum*	Fusobacteria	Fnu	AAL94856	335	AAL94855	233	AAL94854	261	N
	Helicobacter pylori	ε-Proteobacteria	Hpy	AAD08616	327	AAD08617	215	AAD08604	271	N
4	*Salmonella typhimurium*	γ-Proteobacteria	Sty2	AAL19465	338	AAL19466	219	AAL19464	276	N
5	*Streptomyces coelicolor*	Firmicutes; Actinobacteria	Sco	CAB76078	368	CAB76077	240	CAB76076	275	Y
6	*Yersinia pestis*	γ-Proteobacteria	Ype2	CAC90148	328	CAC90149	223	CAC90147	274	N
7	*Sinorhizobium meliloti*	α-Proteobacteria	Sme	CAC47469	358	:CAC47468	221	CAC47467	258	N
	Agrobacterium tumefaciens	α-Proteobacteria	Atu	AAL45281	346	AAK88954	222	AAK88953	259	N
8	*Deinococcus radiodurans*	Thermus-Deinococcus group	Dra	AAF10928	325	AAF10929	218	AAP10931	256	N
				–		–		AAF10930	256	N
9	*Treponema pallidum*	Spirochaetales	Tpa	AAC65110	269	AAC65109	219	AAC65789	268	Y
10	*Pseudomonas aeruginosa*	γ-Proteobacteria	Pael	AAG05738	369	AAG05739	217	AAG07318	259	N
11	*Brucella melitensis*	α-Proteobacteria	Bme	AAL53579	369	AAL53578	230	AAL53580	278	N
				–		–		AAL53135	268	N
	Mesorhizobium loti	α-Proteobacteria	Mlo	NP_105583	365	NP_105582	218	NP_105584	284	N
12	*Neisseria meningitidis*	β-Proteobacteria	Nme	CAB83797	245	CAB83798	228	CAB83799	287	Y
13	*Ralstonia solanacearum*	β-Proteobacteria	Rso	CAD14622	350	CAD14623	217	CAD14624	266	N

14	*Caulobacter crescentus*	α-Proteobacteria	Ccr	AAK24636	332	AAK24635	224	AAK24631	268	Y
	Pseudomonas aeruginosa	γ-Proteobacteria	Pae2	AAGOB888	335	AAG08889	225	AAG08890	260	N
	Xylella fastidiosa	γ-Proteobacteria	Xfa	AAP83685	334	AAF83684	235	AAF83683	261	N
	Xanthomonas axonopodis	γ-Proteobacteria	Xax	AAM38512	335	AAM38511	231	AAM38510	269	N
	Xanthomonas campestris	γ-Proteobacteria	Xca	AAM42900	335	AAM42899	232	AAM42898	266	N
15	*Bacillus anthracis*	Firmicutes; Bacillales	Ban1	NP_654117	346	NP_654116	222	NP654118	270	Y
	Listeria innocua	Firmicutes; Bacillales	Lin2	CAC95545	338	CAC95544	220	CAC95546	273	Y
	Listeria monocytogenes	Firmicutes; Bacillales	Lmo2	CAD008H	338	CAD00810	220	CAD00812	273	Y
16	*Staphylococcus aureus*	Firmicutes; Bacillales	Sau1	BAB56999	341	BAB57000	231	BAB57001	273	Y
	Bacillus anthracis	Firmicutes; Bacillales	Ban2	NP_653454	341	NP_653453	221	NP_653451	268	Y
	Bacillus anthracis	Firmicutes; Bacillales	Ban2	–		–		NP_JS53452	270	Y
	Bacillus halodurans	Firmicutes; Bacillales	Bha	BAB07200	338	BAB07199	218	BAB07198	246	Y
	Bacillus subtilis	Firmicutes; Bacillales	Bsu	CAB 15264	341	CAB 15263	222	CAB 15262	274:	Y
				–		–.		CAB 12739	263	Y
	Listeria innocua	Firmicutes; Bacillales	Lin1	CAC97741	340	CAC97740	224	CAC97739	276	Y
	Listeria monocytogenes	Firmicutes; Bacillales	Lmo1	CAD00497	340	CAD00496	224	CAD00495	276	Y
17	*Staphylococcus aureus*	Firmicutes; Bacillales	Sau2	BAB56624	341	BAB56625	219	BAB56626	280	Y
18	*Corynebacterium glutamicum*	Firmicutes; Actinobacteria	Cg1	NP599870	360	NP599869	225	NP599871	299	Y
19	*Chlamydia pneumoniae*	Chlamydiales	Cpn	AAD 18429	341	AADI8428	221	AAD 18427	272	.N
20	*Clostridium acetobutylicum*	Firmicutes; Bacillus-Clostridium group	Cac	AAK78960	320	AAK78961	218	AAK78962	272	Y
	Campylobacter jejuni	ε-proteobacteria	Cje	CAB73039	336	CAB73038	303	CAB73037	257	N
				–		–		CAB73036	256	N
				–		–		CAB73035	258	N
	Providencia stuartii[a]	γ-Proteobacteria	Pst	–		AAF71397	146	–		
	Mannheimia haemolytica[a]	γ-Proteobacteria	Mha1	–		–		AAA25538	277	Y
			Mha2	–		–		AAA25547	277	Y
			Mha3	–		–		AAA25540	263	Y
	Legionella pneumophila[a]	γ-Proteobacteria	Lpn	–		–		CAA06664	259	Y
	Neisseria gonorrhoeae[a]	β-Proteobacteria	Ngo	–		–		AAF44768	288	Y

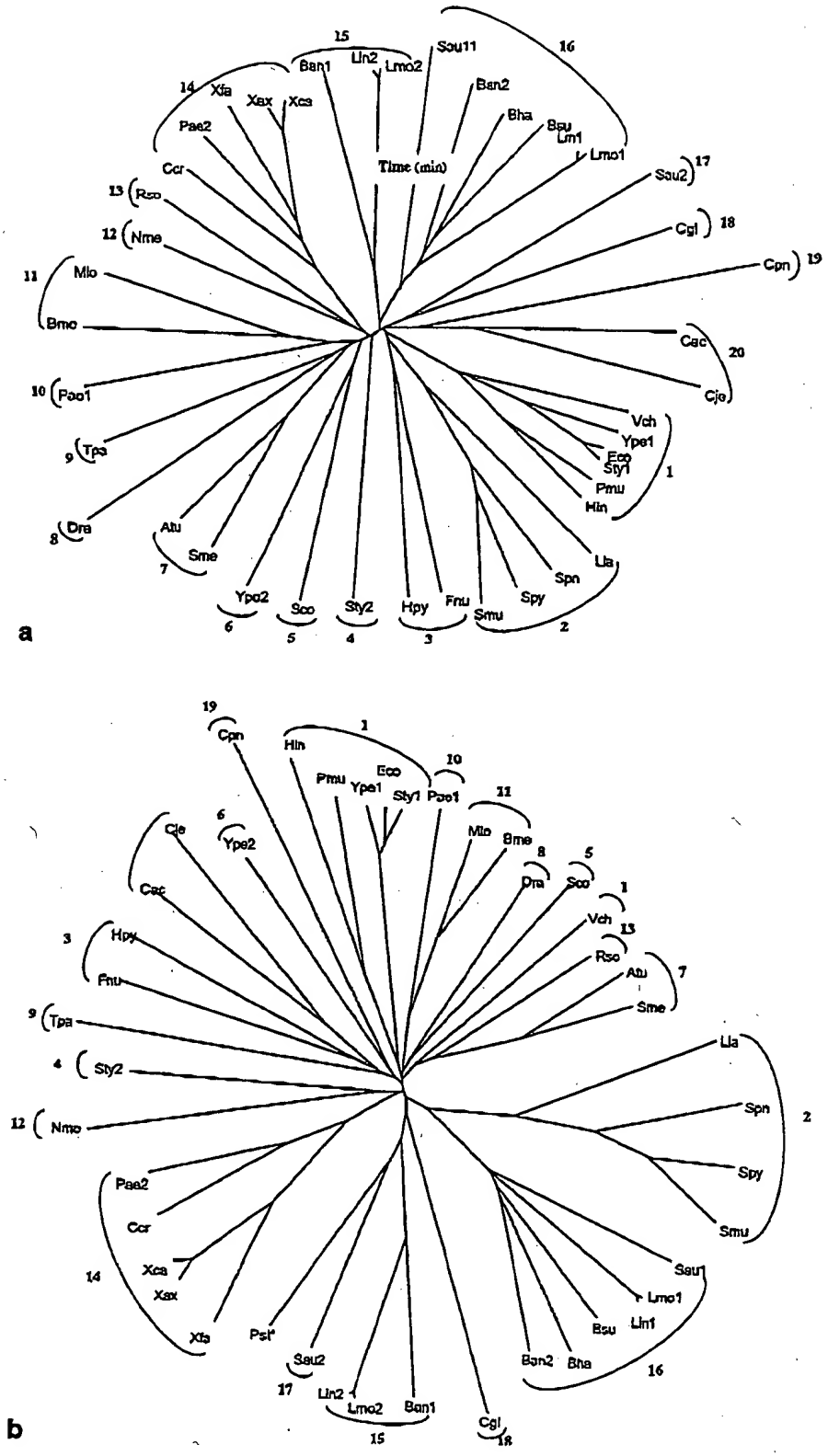
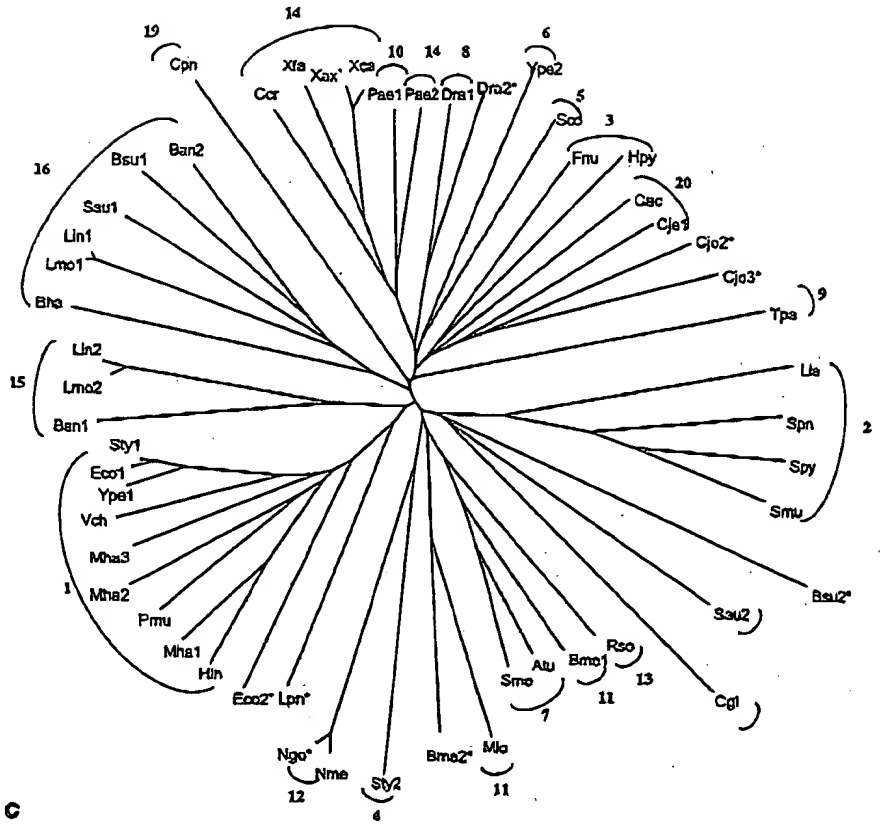


Fig. 4a–c Phylogenetic trees of the three constituents of the MUT family of ABC transporters: a ATP-binding cassette (ABC) constituents, b membrane constituents, c solute binding receptors. The multiple alignments were generated with the Clustal X program (Thompson et al. 1997) using the BLOSUM 62 scoring matrix. The trees are based on the neighbor-joining method and were drawn with the TreeView program (Page 1996). c * refers to a receptor that is not encoded with a gene for an ABC protein or a membrane protein

Fig. 4c



family (TC# 3.A.1.4). We have termed this family the methionine uptake transporter (MUT) family (TC# 3.A.1.23).

We identified the three constituent proteins that comprise MUT family permeases (Table 4). Most organisms having MUT family representation have only one homologue within this family, but a few have two. Organisms with two paralogues include *Salmonella typhimurium*, *Yersinia pestis*, and *Pseudomonas aeruginosa* (all γ-Proteobacteria) as well as *Bacillus anthracis*, *Staphylococcus aureus*, and two species of *Listeria* (all low G+C gram-positive bacteria). No organism has three or more paralogues within the MUT family. The database entry for the Hin membrane protein-encoding gene in *Haemophilus influenzae* was found to be truncated at both the N- and C-termini due to (1) an incorrect initiation codon assignment and (2) a frameshift mutation in the structural gene. This frameshift mutation has been shown to be authentic and not due to a sequencing error. Hin may therefore be encoded by a pseudogene. The reconstructed protein was 198 residues long. The database entries for the Vch and Bha receptors were also found to be erroneous due to incorrect initiation codon assignments (Table 4).

The phylogenetic trees shown in Fig. 4a, b, c for the ABC, membrane and receptor protein constituents of the transporters, respectively, were analyzed according to phylogenetic cluster. Cluster 1 includes the *E. coli* methionine uptake transporter, and only γ-proteobacterial proteins are represented. All of the members of cluster 1 have lipoproteins as receptors. The phylogenies of the proteins follow those of the organisms thereby suggesting orthology. One system, Vch, has its ABC protein and its receptor in cluster 1, but its membrane protein is loosely clustered with the α-proteobacterial proteins of clusters 7 and 13. This may possibly represent an unusual case of shuffling of constituents between systems (Kuan et al. 1995). Only the membrane protein behaved anomalously. However, using PAM distances with the Phylo_win program (Galtier et al. 1996), the membrane constituent Vch clustered together with other cluster 1 proteins. For all complete cluster 1 members, predicted MetJ boxes were found in the promoter regions of the operons as shown in Fig. 5.

Cluster 2 consists of proteins from streptococci and a closely related lactic acid bacterium. These proteins are also probably orthologous to each other.

Cluster 3 proteins from *Helicobacter pylori* and *Fusobacterium nucleatum* always cluster together in spite of the great phylogenetic distance between these two organisms. It seems possible that horizontal transfer had occurred. Therefore, the G+C contents and codon usages of the genes encoding the ABC transporters of these two organisms were compared, but no significant differences were observed relative to those of the genomes. This negative result does not eliminate the possibility of horizontal transfer.

a

Eco	AGACGTCT GGATGCCT TAACATCC		-67
Styl	AGACGTCT GGATGCCT TAACATCC		-68
Ypel	AGCCGTCT AGACGCCT TAACATCC		-64
Vch	AGACGTCT AGACGTAA AAATATCT		-92
Hin	AGCAATCT AGACATCT	-39	
Pmu	GGAAATCT AGACGTCT	-40	

b

Eco	8/8	5/8	4/8
Styl	8/8	5/8	4/8
Ypel	7/8	7/8	4/8
Vch	8/8	6/8	5/8
Hin	5/8	7/8	
Pmu	5/8	8/8	

Fig. 5a, b Conserved MetJ binding sites (Met boxes) found between *abc* and *yaeD* from enterobacteria. a An alignment of proposed Met boxes from *Escherichia coli* (Eco), *Salmonella typhimurium* (Styl), *Yersinia pestis* (Ypel), *Vibrio cholerae* (Vch), *Haemophilus influenzae* (Hin), and *Pasteurella multocida* (Pmu). Protein abbreviations are as indicated in Table 4. The numbers at the *right* indicate the distance from the start codon of *abc*. b The number of matches to the consensus Met box sequence (AGACGTCT) is shown for each predicted Met box

Clusters 4–13 consist of only one or two proteins per cluster. In clusters 7 and 11, the two proteins in each cluster are from α-Proteobacteria, suggesting orthology. However, some of the distantly related proteins belong to closely related organisms (e.g., Sty2 and Ype2; Nme and Rso). This clearly suggests that sequence-divergent primordial proteins resulted from early gene duplication events and that these early paralogues were not transmitted to most of the organisms. Moreover, this conclusion is confirmed by the presence of several lipoproteic receptors from gram-negative organisms (Ccr, Nme, Tpa) although most are soluble (Table 4).

Cluster 14 consists of γ-proteobacterial proteins except for Ccr, which is from an α-proteobacterial species. The clustering patterns in Fig. 4a, c are consistent with orthology, but the clustering of Ccr in Fig. 4b is anomalous, and the same topology was obtained using PAM distances as a model of evolution.

Clusters 15 and 16 clearly represent two sequence-divergent groups of paralogues, both represented in *Bacillus anthracis* and two *Listeria* species. *Staphylococcus aureus* as well as *Bacillus subtilis* and *Bacillus halodurans* encode within their genomes only the second of these homologues (cluster 16). The clustering of Bha in Fig. 4c is anomalous, and this was also observed for the tree drawn using PAM distances. S-boxes have been predicted upstream of the operons encoding all transporters in clusters 15 and 16 with the exception of Bha (data not shown).

Cluster 19 includes a single chlamydial protein although three chlamydial species have been sequenced. In spite of their close phylogenetic relationship, the other two close relatives of *Chlamydophilia pneumoniae* must have lost the corresponding orthologues.

Finally, in cluster 20, Cac and Cjc are always together (Fig. 4a, b, c). Because of the great distance between *Clostridium acetobutylicum* (a gram-positive bacterium) and *Campylobacter jejuni* (a gram-negative ε-proteobacterium), we suggest that horizontal transfer has occurred. However, this assumption can not be confirmed as both organisms have similar overall G+C content, and the G+C content and codon usage patterns of the MUT genes did not prove to be different from those of the complete genomes. An S-box was also predicted in the region of the operon encoding the Cac system.

With the exception of Lla, all receptors from gram-positive organisms were predicted to be lipoproteins. For Lla, a signal peptide with high similarity to those of Spn, Spy, and Smu was found. However, the conserved cysteine residue essential for lipid anchorage was replaced by a glycine residue. This might be explained by a sequencing error or by a single-point mutation since the codons for these two residues differ by only one nucleotide.

Discussion

The results presented here confirm and extend the molecular characterization of the MetD transporter identified earlier in the laboratories of Kadner and Ayling. *abc* and *yaeE* encode, respectively, the ATP-binding cassette (ABC) and membrane proteins of the MetD transporter. YaeC, the receptor encoded with the other components of the transporter, is the major binding protein for both L- and D-methionine. Inhibition studies revealed that the transporter is specific for both methionine isomers and their analogues including *N*-formyl methionine (Table 3; Kadner 1974, 1977). However, the YaeC-related paralogue NlpA (lipoprotein 28) may also exhibit the slight capacity to bind the two isomers of methionine. The *abc-yaeE-yaeC* genes were renamed *metNIQ* by Gal et al. (2002) and Merlin et al. (2002).

We confirmed Kadner's observation that L-methionine effectively competes for D-methionine transport while D-methionine does not strongly compete for L-methionine transport. This led Kadner to suggest that the *metD* locus encodes a component of at least two transport systems but may not encode the initial methionine-binding site (Kadner 1977), a suggestion reiterated by Merlin et al. (2002). However, the data presented here show that MetD is a single transport system with a single major methionine-binding receptor. The difference in inhibition observed for the two isomers is a consequence of the low K_m for L-methionine (75 nM) (Kadner 1974), which is 15-fold lower than that for D-methionine (1.2 μM) (Kadner 1977). We further demonstrate that MetJ is an effective repressor of *metD* expression.

Phylogenetic analyses led to the conclusion that MetD belongs to a new ABC family, which we named the methionine uptake transporter (MUT) family (TC #3.A.1.23). The MUT family is widely represented among bacterial subdivisions. All homologues of each of the three components are of a similar size, and all membrane proteins exhibit five putative transmembrane α-helical segments (TMSs). The overall topology of the trees presented does not fol-

low the phylogenies of the organisms, suggesting that several genetic duplications encoding these systems had occurred early during the evolutionary history of the family. The existence of several sequence-divergent primordial paralogues is likely to explain the topology of the phylogenetic trees. However, no more than two of these paralogues have been transmitted to any currently sequenced organism.

It is interesting to note that *Yersinia pestis* and *Salmonella typhimurium*, two close relatives of *E. coli*, possess two paralogous systems within the MUT family whereas only the receptor is present twice in *E. coli*. The possibility that the common ancestor of these three organisms had two paralogous systems, and that *E. coli* specifically lost the membrane and ABC proteins of one of them is highly unlikely. Ype2 and Sty2 do not cluster with Ype1 and Sty1 although the two receptors, Eco2 and Eco1, are found together in cluster 1. We therefore propose that *nlpA* (encoding Eco2) arose by a much more recent duplication of the *yaeC* gene precursor (encoding Eco1). This conclusion is corroborated by the observation that both Sty2 and Ype2 are not lipoproteins although Eco1 and Eco2 are.

Cluster 1 includes a group of γ-proteobacterial proteins conserved in all three trees. For this cluster, the phylogenies of the proteins agree with those of the organisms. Moreover, we identified MetJ binding sites in the promoter regions of all members of this cluster, and all cluster 1 receptors are predicted to be lipoproteins. These observations strongly suggest that all cluster 1 systems are orthologous methionine transporters, a suggestion confirmed by the presence of the functionally similar MetD transporter in *S. typhimurium* (Ayling and Bridgeland 1972; Ayling et al. 1979; Betteridge and Ayling 1975; Poland and Ayling 1984).

Several members of the MUT family from gram-positive bacteria are likely to transport sulfur compounds. Thus, almost all cluster 15 and 16 constituents (Figs. 4a-c) are encoded in operons regulated by S-boxes (Grundy and Henkin 1998). S-boxes are gram-positive bacterial consensus sequences for the transcriptional control of sulfur metabolism (Grundy and Henkin 1998). The presence of transporters likely to be involved in sulfur acquisition in positions throughout the phylogenetic tree suggests that this entire family may be involved in the transport of organic sulfur compounds. Further experimentation will be required to determine the substrate ranges of the transporters in this family.

The MUT family is of pharmaceutical interest since several members are required for bacterial pathogenicity. *sfbA* of *S. typhimurium* is found in a pathogenicity islet and is essential for infection in a mouse model (Pattery et al. 1999), although its specific contribution to pathogenicity is unknown. *sfbA* was predicted to encode the binding protein of an ABC transporter for iron because its expression was increased under iron-limiting conditions. Despite its regulation by iron, a Fur regulatory binding site was not found close to this operon (Panina et al. 2001). Based on the analyses presented here, we suggest that the Sfb transporter is involved in the uptake of an amino acid or sulfur compound during infection. The *H. influenzae hlpA* gene is not essential for infection, but a mutation in this gene results in reduced invasion in rats (Chanyangam et al. 1991). *Helicobacter pylori* also contains a MUT family transporter (AbcBCD) of unknown function that is required for maximal production of urease, essential for colonization (Hendricks and Mobley 1997). Since many *Yersinia pestis* strains require exogenous methionine (Brubaker 1972), methionine transporters may be drug targets for these organisms.

Acknowledgements This work was supported by NIH grants GM55434 and GM64368 from the National Institute of General Medical Sciences (to MHS). JNF was supported by a Rhône-Alpes Fellowship from France. We thank Mary Beth Hiller for her assistance in the preparation of this manuscript.

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(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 038 970 A2

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
27.09.2000 Bulletin 2000/39

(21) Application number: **00105968.2**

(22) Date of filing: **24.03.2000**

(51) Int. Cl.[7]: **C12P 13/14**, C12N 15/31, C07K 14/345
// (C12P13/14, C12R1:13)

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **25.03.1999 JP 8169399**

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(54) Method for producing L-glutamic acid

(57) L-Glutamic acid is more efficiently produced at lower cost compared with conventional techniques by culturing a coryneform bacterium which has L-glutamic acid-producing ability in a medium to produce and accumulate L-glutamic acid in the medium, and collecting the L-glutamic acid from the medium, wherein an L-glutamic acid uptake system is deleted or decreased in the coryneform bacterium.

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Description

Technical Field

[0001] The present invention relates to an L-glutamic acid-producing bacterium and a method for producing L-glutamic acid by fermentation utilizing the bacterium. L-glutamic acid is an amino acid important for foodstuffs, medical supplies and so forth.

Related Art

[0002] Heretofore, L-glutamic acid has been produced by a fermentation method using coryneform L-glutamic acid-producing bacteria belonging to the genus *Brevibacterium*, or *Corynebacterium* (Amino Acid Fermentation, Gakkai Shuppan Center, pp.195-215, 1986). As such coryneform bacteria, wild strains isolated from nature or mutants thereof are used to improve the productivity.

[0003] Moreover, there have also been disclosed various techniques for promoting L-glutamic acid-producing ability by enhancing genes for enzymes involved in the L-glutamic acid biosynthetic system through recombinant DNA techniques. For example, Japanese Patent Laid-open Publication No. 63-214189 discloses a technique for elevating the L-glutamic acid-producing ability by enhancing a glutamate dehydrogenase gene, isocitrate dehydrogenase gene, aconitate hydratase gene, and citrate synthase gene.

[0004] On the other hand, as for L-threonine, there has been known a technique of disruption of an uptake system of the amino acid in order to increase the production of the amino acid (Okamoto, K. *et al.*, *Biosci. Biotech. Biochem.*, *61* (11), 1877-1882 (1997)).

[0005] For L-glutamic acid, the structure of the genecluster of the uptake system for L-glutamic acid (*gluABCD* operon) in *Corynebacterium glutamicum* has been known (Kronemeyer, W. *et al.*, *J. Bacteriol.*, *177* (5), 1152-1158 (1995)). Moreover, Kronemeyer *et al.* produced a strain in which the L-glutamic acid uptake system encoded by the *gluABCD* operon was deleted, and studied about the excretion of L-glutamic acid using this strain. In this study, they concluded that the excretion of L-glutamic acid of *Corynebacterium glutamicum* did not depend on *gluABCD*, and depended on other uptake and excretion mechanisms. Furthermore, an uptake system of L-glutamic acid other than that encoded by *gluABCD* has also been reported (Burkovski, A. *et al.*, *FEMS Microbiology Letters*, *136*, 169-173 (1996)). These reports suggest that the accumulation amount of L-glutamic acid in the medium is not affected even if at least the L-glutamic acid uptake system encoded by the *gluABCD* operon is deleted. Therefore, it has not been attempted to improve the L-glutamic acid-producing ability by disruption of the uptake system of L-glutamic acid encoded by *gluABCD*.

DETAILED DESCRIPTION OF THE INVENTION

[0006] The object of the present invention is to breed a bacterial strain having high L-glutamic acid-producing ability, and thereby provide a method for more efficiently producing L-glutamic acid at low cost in order to respond to further increase of the demand of L-glutamic acid.

[0007] In order to achieve the aforementioned object, the inventors of the present invention studied assiduously. As a result, they found that an L-glutamic acid-producing bacterium of coryneform bacteria whose *gluABCD* operon was deleted had high L-glutamic acid-producing ability contrary to the suggestion by the aforementioned prior art, and thus accomplished the present invention.

[0008] That is, the present invention provides the followings.

(1) A method for producing L-glutamic acid, comprising the steps of culturing a coryneform bacterium which has L-glutamic acid-producing ability in a medium to produce and accumulate L-glutamic acid in the medium, and collecting the L-glutamic acid from the medium, wherein an L-glutamic acid uptake system is deleted or decreased in the coryneform bacterium.

(2) The method according to (1), wherein the L-glutamic acid uptake system is encoded by the *gluABCD* operon.

(3) The method according to (2), wherein at least one of expression products of the *gluABCD* operon is deleted in the coryneform bacterium.

(4) The method according to (3), wherein at least *gluA* is deleted in the coryneform bacterium.

(5) The method according to (4), wherein all of *gluA*, *gluB*, *gluC* and *gluD* are deleted in the coryneform bacterium.

[0009] According to the present invention, L-glutamic acid can be produced at a higher yield compared with conventional techniques.

[0010] For the present invention, the term "L-glutamic acid-producing ability" refers to an ability of a coryneform

bacterium used for the present invention to accumulate L-glutamic acid in a medium when the bacterium is cultured in the medium.

[0011] Hereafter, the present invention will be explained in detail.

[0012] The coryneform bacterium used for the present invention is a coryneform bacterium having L-glutamic acid-producing ability, in which an L-glutamic acid uptake system is deleted or decreased.

[0013] Bacteria belonging to the genus *Corynebacterium* as referred to herein are a group of microorganisms defined in Bergey's Manual of Determinative Bacteriology, 8th Ed., p. 599 (1974). The bacteria are aerobic, Gramposi-tive, nonacid-fast bacilli not having the ability to sporulate, and include bacteria which had been classified as bacteria belonging to the genus *Brevibacterium* but have now been unified into the genus *Corynebacterium* [see Int. *J. Syst. Bacteriol.*, *41*, 255 (1981)] and also include bacteria of the genus *Brevibacterium* and Microbacterium which are closely related to the genus *Corynebacterium*. Examples of coryneform bacteria preferrably used for producing L-glutamic acid include the followings.

Corynebacterium acetoacidophilum
Corynebacterium acetoglutamicum
Corynebacterium alkanolyticum
Corynebacterium callunae
Corynebacterium glutamicum
Corynebacterium lilium (Corynebacterium glutamicum)
Corynebacterium melassecola
Corynebacterium thermoaminogenes
Corynebacterium herculis
Brevibacterium divaricatum (*Corynebacterium glutamicum*)
Brevibacterium flavum (*Corynebacterium glutamicum*)
Brevibacterium immariophilum
Brevibacterium lactofermentum (*Corynebacterium glutamicum*)
Brevibacterium roseum
Brevibacterium saccharolyticum
Brevibacterium thiogenitalis
Brevibacterium ammoniagenes (*Corynebacterium ammoniagenes*)
Brevibacterium album
Brevibacterium cerinum
Microbacterium ammoniaphilum

[0014] Specifically, the following strains of these bacteria are exemplified:

Corynebacterium acetoacidophilum ATCC13870
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium alkanolyticum ATCC21511
Corynebacterium callunae ATCC15991
Corynebacterium glutamicum ATCC13020, 13032, 13060
Corynebacterium lilium (*Corynebacterium glutamaicum*) ATCC15990
Corynebacterium melassecola ATCC17965
Corynebacterium thermoaminogenes AJ12340 FERN BP-1539)
Corynebacterium herculis ATCC13868
Brevibacterium divaricatum (*Corynebacterium glutamicum*) ATCC14020
Brevibacterium flavum (*Corynebacterium glutamicum*) ATCC13826, ATCC14067
Brevibacterium immariophilum ATCC14068
Brevibacterium lactofermentum (*Corynebacterium glutamicum*) ATCC13665, ATCC13869
Brevibacterium roseum ATCC13825
Brevibacterium saccharolyticum ATCC14066
Brevibacterium thiogenitalis ATCC19240
Corynebacterium ammoniagenes (*Brevibacterium* ammoniagenes) ATCC6871
Brevibacterium album ATCC15111
Brevibacterium cerinum ATCC15112
Microbacterium ammoniaphilum ATCC15354

[0015] The aforementioned deletion or decrease of an L-glutamic acid uptake system of coryneform bacteria can

be attained by mutating or disrupting a gene coding for the uptake system using a mutagenesis treatment or a genetic recombination technique. The term " L-glutamic acid uptake system is deleted or decreased" means to make the uptake system not function normally, and it includes that at least one of proteins constituting the uptake system is deleted or the activity of the protein is decreased, and that two or more of the proteins are deleted or the activities of the proteins are decreased. Further, the term "a protein is deleted" used herein include both of a case where the protein is not expressed at all, and a case where a protein that does not normally function is expressed.

[0016] The gene coding for the L-glutamic acid uptake system can be disrupted by gene substitution utilizing homologous recombination. A gene on a chromosome of a coryneform bacterium can be disrupted by transforming the coryneform bacterium with DNA containing a gene modified by deleting its internal sequence (deletion type gene) so that the uptake system should not function normally to cause recombination between the deletion type gene and a normal gene on the chromosome. Such gene disruption utilizing homologous recombination has already been established, and there have been known methods therefor utilizing linear DNA, plasmid containing a temperature sensitive replication origin and so forth. A method utilizing a plasmid containing a temperature sensitive replication origin is preferred.

[0017] As a gene coding for an L-glutamic acid uptake system, the *glUABCD* operon has been known (Kronemeyer, W. *et al.*, *J. Bacteriol.*, 177 (5), 1152-1158 (1995)). Moreover, an L-glutamic acid uptake system other than that encoded by the *gluABCD* operon has also been known (Burkovski, A. *et al.*, *FEMS Microbiology Letters*, 136, 169-173 (1996)). Although any of such genes may be disrupted, it is preferred that the uptake system encoded by the *gluABCD* operon is disrupted.

[0018] Because the nucleotide sequence of this operon has been known (GenBank/EMBL/DDBJ Accession X81191), this operon or each structural gene in the *gluABCD* operon can be isolated from chromosome DNA of coryneform bacteria by PCR using primers produced based on the nucleotide sequence. A certain region can be excised from the thus obtained gene fragment with one or more restriction enzymes, and at least a part of a coding region or an expression control sequence such as promoter can be deleted to prepare a deletion type gene.

[0019] Further, a deletion type gene can also be obtained by performing PCR using primers designed so that a part of a gene should be deleted. For example, by using the primers having the nucleotide sequences shown as SEQ ID NOS: 1 and 2 in Sequence Listing, a *gluD* gene having a deletion of a part of 5' sequence can be obtained. Further, by using the primers having the nucleotide sequences shown as SEQ ID NOS: 3 and 4, a *gluA* gene having a deletion of a part of 3' sequence can be obtained. When gene substitution is performed by using the deletion type *gluA* gene or *gluD* gene obtained by using those primers, the *gluA* gene or the *gluD* gene can be disrupted. Further, if these deletion type *gluA* gene and deletion type *gluD* gene are ligated, and the obtained fusion gene is used for gene substitution, all of *gluA*, *gluB*, *gluC* and *gluD* can be disrupted. Furthermore, when PCR is performed by using a plasmid containing a *gluA* gene that has been obtained by using primers having the nucleotide sequences shown as SEQ ID NOS: 3 and 5 as a template, and primers having the nucleotide sequences shown as SEQ ID NOS: 6 and 7, and the amplification product is cyclized, there can be obtained a plasmid containing *gluA* gene including a deletion of an internal sequence and having 5' region and 3' region ligated in-flame. When gene substitution is performed by using this plasmid, only the *gluA* gene can be disrupted.

[0020] Moreover, primers other than those exemplified above can also be designed by the methods well known to those skilled in the art, and an arbitrary structural gene in the *gluABCD* operon can be disrupted by using such primers. Alternatively, the uptake system can be deleted by deleting an expression control sequence of the *gluABCD* operon such as a promoter so that the gene cannot be expressed.

[0021] While the gene substitution of the *gluABCD* gene-cluster (henceforth referred to simply as "*gluABCD* gene") will be explained below, an arbitrary structural gene or expression control sequence can be similarly deleted.

[0022] The *gluABCD* gene on a host chromosome can be replaced with a deletion type *gluABCD* gene as follows. That is, a recombinant DNA is constructed by insertion of a temperature sensitive replication origin, a deletion type *gluABCD* gene and a maker gene expressing resistance to a drug such as chloramphenicol, tetracycline and streptomycin, and a coryneform bacterium is transformed with this recombinant DNA. Then, the transformant strain can be cultured at a temperature at which the temperature sensitive replication origin does not function, and then cultured in a medium containing a corresponding drug to obtain a transformant strain in which the recombinant DNA is integrated into chromosomal DNA.

[0023] In such a strain in which a recombinant DNA is integrated into a chromosome as described above, recombination of the *gluABCD* gene sequence originally present on the chromosome has been caused, and two fusion genes of the chromosomal *gluABCD* gene and the deletion type *gluABCD* gene are inserted into the chromosome, between which the other parts of the recombinant DNA (the vector protion, temperature sensitive replication origin, and drug resistance marker) are present. Therefore, because the normal *gluABCD* gene is dominant in this state, the transformant strain expresses the L-glutamic acid uptake system.

[0024] Then, in order to leave only the deletion type *gluABCD* gene on the chromosome DNA, one copy of the *gluABCD* gene is eliminated from the chromosome DNA together with the vector protion (including temperature sensitive replication origin and drug resistance marker) by recombination of the two *gluABCD* gene. Upon the recombination,

the normal *gluABCD* gene may be left on the chromosome DNA and the deletion type *gluABCD* gene may be excised, or the deletion type *gluABCD* gene may be left on the chromosome DNA and the normal *gluABCD* gene may be excised. In both of the cases, the excised DNA is retained on the plasmid in a cell when the cell is cultured at a temperature at which the temperature sensitive replication origin functions. Such DNA on the plasmid is eliminated from the cell when the cell is cultured at a temperature at which the temperature sensitive replication origin does not function. It can be confirmed which genes are left on the chromosome DNA by investigating the structure of the *gluABCD* gene on the chromosome by PCR, hybridization or the like.

[0025] When such a *gluABCD* gene-disrupted strain produced as described above is cultured at a temperature at which the temperature sensitive replication origin functions (for example, low temperature), the *gluABCD* gene will be retained in its cell. When it is cultured at a temperature at which the temperature sensitive replication origin does not function (for example, elevated temperature), the *gluABCD* gene will be eliminated from the cell.

[0026] Examples of the plasmid which has a temperature sensitive replication origin functioning in coryneform bacterial cells include, pHS4, pHSC4, pHS22, pHSC22, pHS23, pHSC23 (as for these, see Japanese Patent Publication (Kokoku) No. 7-108228) and so forth. These temperature sensitive plasmids can autonomously replicate at a temperature of about 10-32°C, but cannot autonomously replicate at a temperature of about 34°C or higher in a coryneform bacterial cell.

[0027] After the *gluABCD* gene on the chromosome are disrupted as described above, the genes-disrupted strain is preferably introduced with *recA*$^-$ because such introduction of *recA*$^-$ prevents the *gluABCD* gene on the plasmid from being integrated again into the chromosome again during culture at a low temperature.

[0028] The coryneform bacterium used for the present invention may have enhanced activity of an enzyme for catalyzing the biosynthesis of L-glutamic acid in addition to the deletion or decrease of L-glutamic acid uptake system. Illustrative examples of the enzyme for catalyzing the biosynthesis of L-glutamic acid include glutamate dehydrogenase, glutamine synthetase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, citrate synthase, pyruvate carboxylase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate synthase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, fructose bisphosphate aldolase, phosphofructokinase, glucose phosphate isomerase and the like.

[0029] Further, in the coryneform bacterium used for the present invention, an enzyme that catalyzes a reaction for generating a compound other than L-glutamic acid by branching off from the biosynthetic pathway of L-glutamic acid may be decreased of or deleted. Illustrative examples of the enzyme which catalyzes a reaction for generating a compound other than L-glutamic acid by branching off from the biosynthetic pathway of L-glutamic acid include a-ketoglutarate dehydrogenase, isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetohydroximate synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrroline dehydrogenase and the like.

[0030] Furthermore, by introducing a thermosensitive mutation for a biotin activity inhibiting substance such as surface active agents into a coryneform bacterium having L-glutamic acid-producing ability, the bacterium becomes to be able to produce L-glutamic acid in a medium containing an excessive amount of biotin in the absence of a biotin activity inhibiting substance (see WO96/06180). As such a coryneform bacterium, the Brevibacterium lactofermentum AJ13029 strain disclosed in WO96/06180 can be mentioned. The AJ13029 strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology on September 2, 1994, and received an accession number of FERM P-14501. Then, its was transferred to an international deposition under the provisions of the Budapest Treaty on August 1, 1995, and received an accession number of FERM BP-5189.

[0031] When a coryneform bacterium having L-glutamic acid-producing ability, in which the L-glutamic acid uptake system is deleted or decreased, is cultured in a suitable medium, L-glutamic acid is accumulated in the medium. Because of the deletion or decrease of L-glutamic acid uptake system in the coryneform bacterium used for the present invention, L-glutamic acid secreted from the cell is prevented from being taken up again into the cell. As a result, the accumulation amount of L-glutamic acid in the medium is increased. According to the method of the present invention, improvement of interval yield (ratio of the accumulation amount of L-glutamic acid to the consumption of saccharides in a certain period of cultivation) can be expected when L-glutamic acid concentration in the medium becomes high. In particular, when a highly productive strain that shows a high L-glutamic acid concentration in a medium during fermentation is used, a marked effect can be obtained.

[0032] The medium used for producing L-glutamic acid by utilizing the microorganism of the present invention is a usual medium that contains a carbon source, a nitrogen source, inorganic ions and other organic trace nutrients as required. As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose, starch hydrolysate and molasses or the like; alcohols such as ethanol, inositol; or organic acids such as acetic acid, fumaric acid, citric acid and succinic acid or the like.

[0033] As the nitrogen source, there can be used inorganic ammonium salts such as ammonium sulfate, ammonium nitrate, ammonium chloride, ammonium phosphate and ammonium acetate, ammonia, organic nitrogen such as peptone, meat extract, yeast extract, corn steep liquor and soybean hydrolysates, ammonia gas, aqueous ammonia

and so forth.

[0034] As the inorganic ions (or sources thereof), added is a small amount of potassium phosphate, magnesium sulfate, iron ions, manganese ions and so forth. As for the organic trace nutrients, it is desirable to add required substances such as vitamin B1, yeast extract and so forth in a suitable amount as required.

[0035] The culture is preferably performed under an aerobic condition attained by shaking, stirring for aeration or the like for 16 to 72 hours. The culture temperature is controlled to be at 30°C to 45°C, and pH is controlled to be 5 to 9 during the culture. For such adjustment of pH, inorganic or organic acidic or alkaline substances, ammonia gas and so forth can be used.

[0036] Collection of L-glutamic acid from fermentation broth can be attained by, for example, methods utilizing ion exchange resin, crystallization and so forth. Specifically, L-glutamic acid can be adsorbed or isolated by an anion exchange resin, or crystallized by neutralization.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037]

Fig. 1 shows the scheme of construction of a plasmid pTΔAD for disrupting *gluABCD* gene.
Fig. 2 shows the scheme of construction of a plasmid pTΔA for disruption of *gluA*.

BEST MODE FOR CARRYING OUT THE INVENTION

[0038] The present invention will be further specifically explained hereinafter with reference to the following examples.

(1) Construction of plasmid for disruption of *gluABCD* gene

[0039] In order to create a *gluABCD* gene-disrupted strain of coryneform bacterium by homologous recombination using a temperature sensitive plasmid, a plasmid for disruption of the *gluABCD* gene was constructed.

[0040] First, a deletion type *gluABCD* gene was constructed by cloning a *gluD* gene having a deletion of 5' sequence, and ligating it with a *gluA* gene having a deletion of 3' sequence. Specifically, a fragment of about 300 bp from a *Bam*HI site present in *gluD* to a site about 270 bp downstream from *gluD* was amplified from chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869, which was a wild-type strain of coryneform bacterium, by PCR utilizing oligonucleotides having the nucleotide sequences represented as SEQ ID NOS: 1 and 2 as primers. This amplified fragment was digested with *Bam*HI and *xba*I, and the obtained fragment was ligated to pHSG299 (produced by Takara Shuzo) digested with *Bam*HI and *Xba*I using T4 ligase (produced by Takara Shuzo) to obtain a plasmid pHSGΔgluD.

[0041] Then, a fragment of about 300 bp from a site about 180 bp upstream from *gluA* to the *Bam*HI site present in *gluA* was amplified from chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 by PCR utilizing oligonucleotides having the nucleotide sequences represented as SEQ ID NOS: 3 and 4 as primers. This amplified fragment was digested with *Eco*RI and *Bam*HI, and the obtained fragment was ligated to pHSGΔgluD digested with *Eco*RI and *Bam*HI by using T4 ligase to obtain a plasmid pHSGΔgluAD. This plasmid had a structure in which *gluB* and *gluC* were deleted, and parts of *gluA* and *gluD* were ligated.

[0042] Then, in order to make pHSGΔgluAD autonomously replicable in coryneform bacteria, a temperature sensitive replication origin derived form a plasmid autonomously replicable in coryneform bacteria was introduced into the unique *Hinc*II cleavage site in pHSGΔgluAD. Specifically, the following procedure was used.

[0043] A plasmid pHSC4 containing a temperature sensitive replication origin (see Japanese Patent Laid-open Publication (Kokai) No. 5-7491) was digested with *Bam*HI and *Kpn*I. The both termini of the obtained DNA fragment was blunt-ended using Blunting Kit (produced by Takara Shuzo), ligated with a *Kpn*I linker (produced by Takara Shuzo), and then allowed to cause self-ligation to obtain pKCT4. pHSC4 was a plasmid obtained as follows. That is, a DNA fragment containing a replication origin was excised from a plasmid pAJ1844 (see Japanese Patent Laid-open Publication (Kokai) No. 58-216199), which had a replication origin derived from pHM1519 (K. Miwa et *al.*, *Agric. Biol. Chem.*, 48, 2901-2903 (1984), Japanese Patent Laid-open Publication (Kokai) No. 58-77895), and ligated to a plasmid for *Escherichia coli*, pHSG298, to obtain a shuttle vector pHK4. This pHK4 was treated with hydroxylamine to obtain a plasmid pHS4 modified to be temperature sensitive. The temperature sensitive replication origin was excised from pHS4, and ligated to pHSG398 to obtain pHSC4. *Escherichia coli* AJ12571 harboring pHSC4 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology on October 11, 1990, and received the accession number of FERM P-11763. Then, it was transferred to international deposit under the Budapest Treaty on August 26, 1991, and received the accession number of FERM BP-3524.

[0044] pKCT4 produced as described above had a structure where the replication origin derived from pHM1519

modified to be temperature sensitive was inserted into *Kpn*I site of pHSG399. A fragment containing a temperature sensitive replication origin was obtained by digesting pKCT4 with *Kpn*I, blunt-ended by using Blunting Kit (produced by Takara Shuzo), and ligated to pHSGΔgluAD digested with *Hinc*II to obtain pTΔAD (Fig. 1).

(2) Construction of plasmid for disruption of *gluA* gene

[0045] In order to create a coryneform bacterium in which only the *gluA* gene was disrupted, a plasmid for disruption of the *gluA* gene was constructed.

[0046] A DNA fragment of about 1500 bp containing the *gluA* gene was amplified from chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 by PCR utilizing oligonucleotides having the nucleotide sequences shown as SEQ ID NOS: 3 and 5 as primers, and the amplified fragment was digested with *Eco*RI, and ligated to pHSG299 (produced by Takara Shuzo) digested with *Eco*RI by using T4 ligase (produced by Takara Shuzo) to obtain a plasmid pHSGgluA.

[0047] Then, the 5' region and the 3' region of the *gluA* gene and the vector segment, except for the internal region of the *gluA* gene, were amplified by PCR utilizing oligonucleotides having the nucleotide sequences shown as SEQ ID NOS: 6 and 7 as primers, and pHSGgluA as a template. The aforementioned primers were designed so that it should contain a *Bgl*II recognition sequence. The amplified fragment was digested with *Bgl*II, and allowed to cause self-ligation in the presence of T4 ligase to obtain a plasmid pHSGΔgluA. This plasmid contained deletion of about 250 bp of internal sequence among the about 730 bp open reading frame of *gluA*, and had a structure where the 5' region and the 3' region were ligated in-frame.

[0048] Then, in order to make pHSGΔgluA autonomously replicable in coryneform bacteria, a temperature sensitive replication origin derived from a plasmid autonomously replicable in coryneform bacteria was introduced into the unique *Kpn*I cleavage site in pHSGΔgluA. Specifically, pKCT4 was digested with *Kpn*I to obtain a DNA fragment containing a replication origin, and the obtained fragment was inserted into *Kpn*I site of pHSGΔgluA to obtain pTΔA (Fig. 2).

(3) Creation of *gluABCD* gene-disrupted strain and *gluA* gene-disrupted strain

[0049] The plasmids for disrupting genes obtained as described above, pTΔAD and pTΔA, were introduced into a wild-type strain, *Brevibacterium lactofermentum* ATCC13869 strain, by using the electric pulse method to obtain ATCC13869/pTΔAD and ATCC13869/pTΔA. Gene disruption was performed by using these transformant strains.

[0050] Specifically, ATCC13869/pTΔAD and ATCC13869/pTΔA were cultured at 25°C in CM2B broth for 24 hours with shaking, and inoculated to CM2B medium containing 25 μg/ml of kanamycin. Strains into which the plasmids were introduced were obtained as strains that formed colonies at 34°C, at which temperature the temperature sensitive replication origin did not function. Then, the strains that became sensitive to kanamycin at 34°C were obtained by the replica method. Chromosome DNA of these sensitive strains was obtained in a conventional manner. The structures of the *gluABCD* gene and the *gluA* gene on the chromosome was examined by PCR and sequencing to confirm that these genes should be replaced with those of the deletion type, and the strains containing the deletion type genes were designated as ΔAD strain and ΔA strain, respectively.

[0051] The ΔAD strain and the ΔA strain were given with private numbers of AJ13587 and AJ13588, respectively, and deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (postal code: 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on March 23, 1999, and received accession numbers of FERM P-17327 and FERM P-17328, respectively, and then, transferred from the original deposit to international deposit based on Budapest Treaty on February 14, 2000, and have been deposited as deposit numbers of FERM BP-7028 and FERM BP-7029, respectively.

(4) Evaluation of L-glutamic acid-producing ability of strains ΔAD and ΔA

[0052] Culture of the strains ATCC13869, ΔAD and ΔA for the production of L-glutamic acid was performed as follows. These strains that had been refreshed by culture in a CM2B plate medium were cultured in two kinds of mediums, a medium containing 80 g of glucose, 1 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $MnSO_4 \cdot 7H_2O$, 15 ml of soybean hydrolysate solution, 200 μg of thiamin hydrochloride, 3 μg of biotin, and 50 g of $CaCO_3$ in 1 L of deionized water (prepared at pH 8.0 by using KOH), and a medium further containing 50 g/L of L-glutamic acid in the foregoing medium, at 31.5°C. After the cultivation, the amounts of accumulated L-glutamic acid in the mediums, and absorbance at 620 nm of the mediums diluted 51 times were measured. The results obtained for the medium with no addition of L-glutamic acid were shown in Table 1. The results obtained for the medium added with L-glutamic acid were shown in Table 2.

Table 1

Strain	OD_{620}	L-glutamic acid (g/L)	Yield (%)
ATCC 13869	0.937	40.8	50.4
ΔAD	1.127	36.3	44.9
ΔA	0.766	44.3	54.8

Table 2

Strain	OD_{620}	L- glutamic acid (g/L)*	Yield (%)
ATCC 13869	0.845	29.5	43.9
ΔAD	0.927	30.0	44.6
ΔA	0.749	32.0	47.6

* The amounts do not include the L-glutamic acid added to the medium.

[0053] These results show that the accumulation amount and yield of L-glutamic acid were improved for both of the ΔA strain and the ΔAD strain when the medium contained L-glutamic acid at a high concentration. Further, the yield of L-glutamic acid was improved by the ΔA strain even in the medium not containing L-glutamic acid.

SEQUENCE LISTING

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Claims

1. A method for producing L-glutamic acid, comprising the steps of culturing a coryneform bacterium which has L-glutamic acid-producing ability in a medium to produce and accumulate L-glutamic acid in the medium, and collecting the L-glutamic acid from the medium, wherein an L-glutamic acid uptake system is deleted or decreased in the coryneform bacterium.

2. The method according to claim 1, wherein the L-glutamic acid uptake system is encoded by the *glUABCD* operon.

3. The method according to claim 2, wherein at least one of expression products of the *gluABCD* operon is deleted in the coryneform bacterium.

4. The method according to claim 3, wherein at least *gluA* is deleted in the coryneform bacterium.

5. The method according to claim 4, wherein all of *gluA*, *gluB*, *gluC* and *gluD* are deleted in the coryneform bacterium.

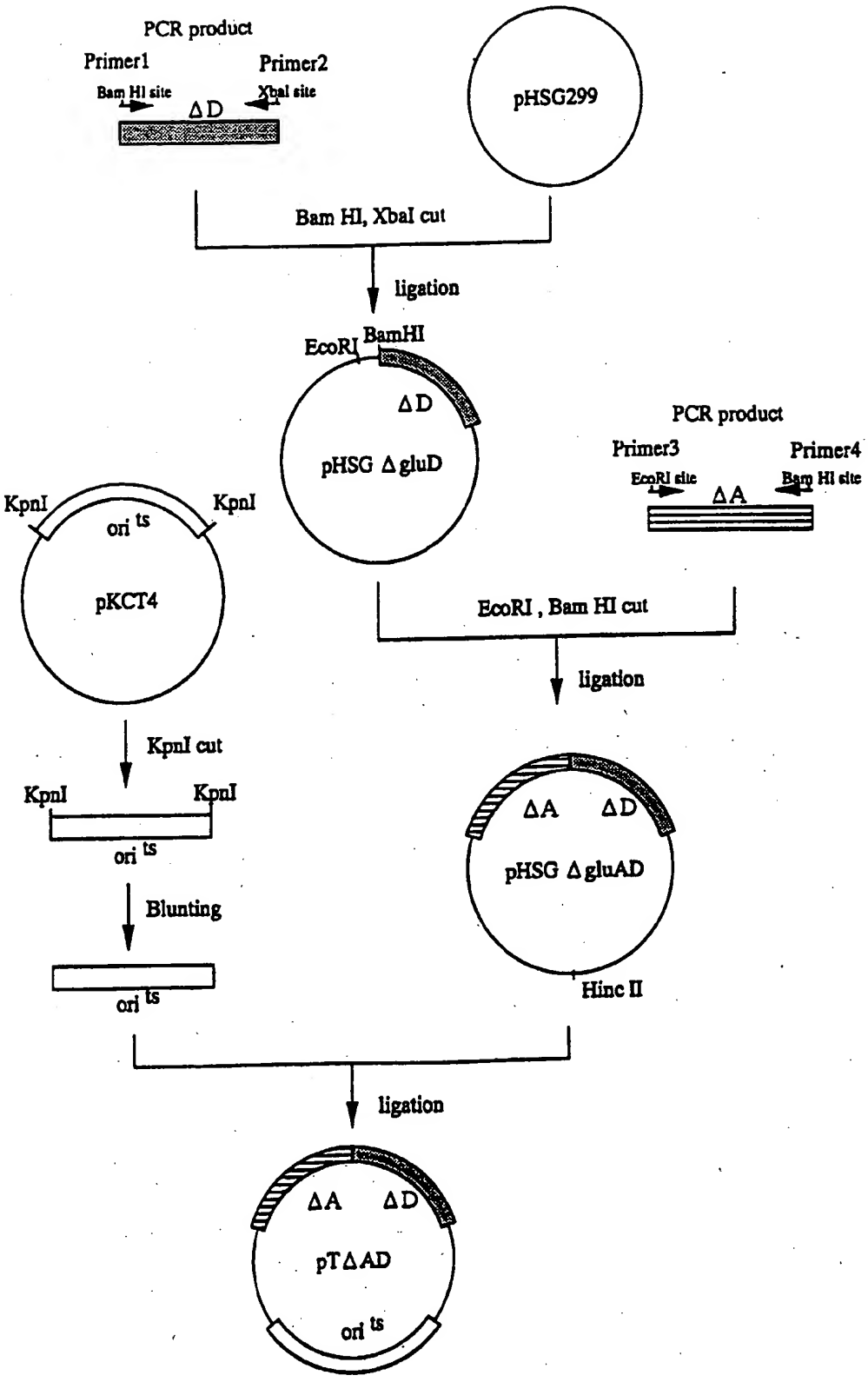
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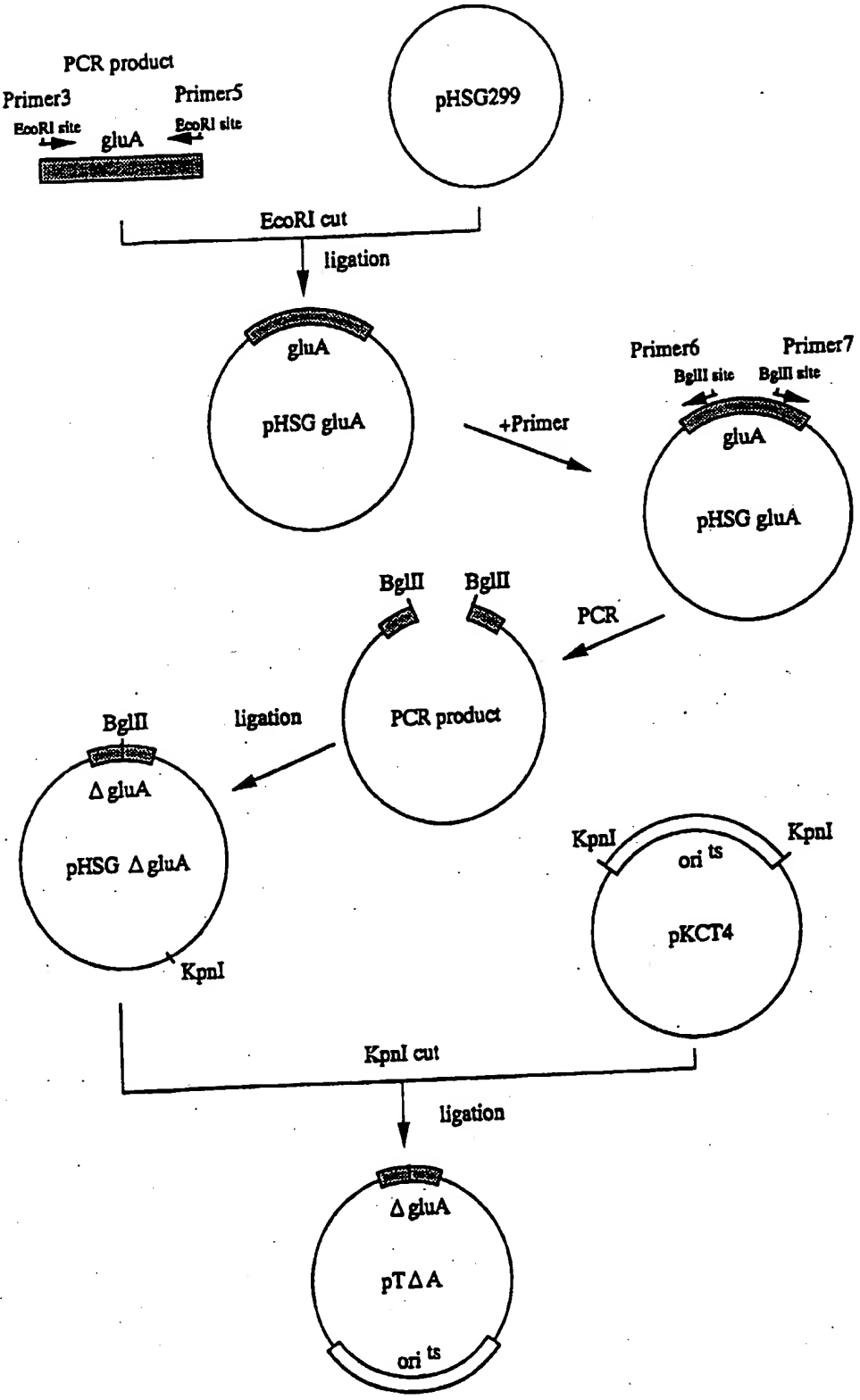
Fig. 1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ori ts

Fig. 2

(12) PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. AU 199719218 B2
(10) Patent No. 724536

(54) Title

Process for the microbial production of amino acids by boosted activity of export carriers

(51)[6] International Patent Classification(s)
C12N 015/00

(21) Application No: 199719218

(22) Application Date: 1996.12.18

(87) WIPO No: WO97/23597

(30) Priority Data

(31) Number	(32) Date	(33) Country
19548222	1995.12.22	DE

(43) Publication Date : 1997.07.17
(43) Publication Journal Date : 1997.09.11
(44) Accepted Journal Date : 2000.09.21

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(51) Internationale Patentklassifikation 6:
C12N 15/00
SEARCH QUALITY ASSURANCE
A2

(11) Internationale Veröffentlichungsnummer: WO 97/23597

(43) Internationales Veröffentlichungsdatum: 3. Juli 1997 (03.07.97)

(21) Internationales Aktenzeichen: PCT/DE96/02485

(22) Internationales Anmeldedatum: 18. December 1996 (18.12.96)

(30) Prioritätsdaten:
195 48 222.0 22. December 1995 (22.12.95) DE

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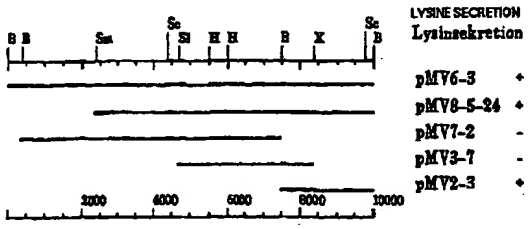
(81) Bestimmungsstaaten: AU, BR, CA, CN, JP, KR, MX, RU, US, europäisches Patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Veröffentlicht
Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.

(54) Title: PROCESS FOR THE MICROBIAL PRODUCTION OF AMINO ACIDS BY BOOSTED ACTIVITY OF EXPORT CARRIERS

(54) Bezeichnung: VERFAHREN ZUR MIKROBIELLEN HERSTELLUNG VON AMINOSÄUREN DURCH GESTEIGERTE AKTIVITÄT VON EXPORTCARRIERN



(57) Abstract

The invention pertains to a process for the microbial production of amino acids. The process in question involves boosting the export carrier activity and/or export gene expression of a micro-organism which produces the desired amino acid. According to the invention, it was found that a single specific gene is responsible for the export of a given amino acid, and on that basis a process for the microbial production of amino acids, involving the controlled boosting of the export gene expression and/or export carrier activity of a micro-organism which produces the amino acid in question, has been developed for the first time. The boosted expression or activity of the export carrier resulting from this process increases the secretion rate and thus increases transport of the desired amino acid.

PROCESS FOR THE MICROBIAL PRODUCTION OF AMINO ACIDS BY BOOSTED ACTIVITY OF EXPORT CARRIERS

The invention relates to a process for the microbial production of amino acids according to claims 1 to 20, export genes according to claims 21 to 26, regulator genes according to claims 29 and 30, vectors according to claims 31 to 33, transformed cells according to claims 34 to 40, membrane proteins according to claims 41 and 42 and uses according to claim 43 and 48.

Amino acids are of high economical interest and there are many applications for the amino acids: for example, L-lysine as well as L-threonine and L-tryptophan are needed as feed additives, L-glutamate as seasoning additive, L-isoleucine, and L-tryosine in the pharmaceutical industry, L-arginine and L-isoleucine as medicine or L-glutamate and L-phenylalanine as a starting substance for the synthesis of fine chemicals.

A preferred method for the manufacture of these different amino acids is the biotechnological manufacture by means of microorganisms; since, in this way, the biologically effective and optically active form of the respective amino acid is directly obtained and simple and inexpensive raw materials can be used. As microorganisms, for example, Carynebacterium glutamicum and its relatives ssp. flavum and ssp lactofermentum (Liebl et al; Int. J-System Bacteriol (1991) 41:255-260) as well as Escherichia coli and related bacteria can be used.

However, these bacteria produce the amino acids only in the amounts needed for their growth such that no excess amino acids are generated and are available. The reason for this is that in the cell the biosynthesis of the amino acids is con-

trolled in various ways. As a result, different methods of increasing the formation of products by overcoming the control mechanisms are already known. In these processes, for example, amino acid analogs are utilized to render the control of the biosynthesis ineffective. A method is described, for example, wherein Coarynebacterium strains are used which are resistant to L-tyrosine and L-phenylalanine analogs (JP 19037/1976 and 39517/1978). Also methods have been described in which bacteria resistant to L-lysine and also to L-threonine analogs are used in order to overcome the control mechanisms (EP 0 205 849 B1, UK patent application GB 2 152 509 A).

Furthermore, microorganisms constructed by recombinant DNA techniques are known wherein the control of the biosynthesis has also been eliminated by cloning and expressing the genes which code for the key enzymes which cannot be feed-back inhibited any more. For example, a recombinant L-lysinee producing bacterium with plasmid-coded feedback-resistant aspartate kinase is known (EP 0381527). Also, a recombinant L-phenylalanine producing bacterium with feedback resistant prephenate dehyrodgenase has been described (JP 124375/1986; EP 0 488 424). In addition, increased amino acid yields have been obtained by overexpression of genes which do not code for feedback-sensitive enzymes of the amino acids synthesis. For example, the lysine formation is improved by increased synthesis of the dihydrodipicolinate synthase (EP 0 197 335). Also, the threonine formation is improved by increased synthesis of threonine dehydratase (EP 0 436 886 A1).

Further experiments for increasing the amino acid production aim at an improved generation of the cellular primary metabolites of the central metabolism. In this connection, it is known that the overexpression of the transketolase achieved by recombinant techniques improve the product generation of L-tryptophan, L-tyrosine or L-phenalanine (EP 0 600 463 A2).

Furthermore, the reduction of the phosphenol pyruvate carboxylase activity in Carynebacterium provides for an improvement in the generation of aromatic amino acids (EP 0 331 145).

All these attempts to increase the productivity have the aim to overcome the limitation of the cytosolic synthesis of the amino acids. However, as a further limitation basically also the export of the amino acids formed in the interior of a cell into the culture medium should be taken into consideration. As a result, it has been tried to improve this export and, consequently, the efficiency of the amino acid production. For example, the cell permeability of the Carynebacterium has been increased by biotin deficiency, detergence or penicillin treatment. However, these treatments were effective exclusively in the production of glutamate, whereas the synthesis of other amino acids could not be improved in this manner. Also, bacteria strains have been developed in which the activity of the secretion system is increased by chemical or physical mutations. In this way, for example, a Corynebacterium glutamicum strain has been obtained which has an improved secretion activity and is therefore especially suitable for the L-Lysine production. (DE 02 03 320).

Altogether, the attempts to increase the secretion of amino-acids formed within the cell have all in common that an increase efflux of amino acids on the basis of the selected non-directed and non-specific methods could be achieved only accidentally.

Solely in the German patent application No. 195 23 279.8-41, a process is described which provides for a well-defined increase of the secretion of amino acids formed internally in a cell by increasing the expression of genes coding for the import of amino acids. The understanding on which this process was based, that is, the cell utilizes import proteins for the export of amino acids as well as the fact that by nature micro-

organisms do not generate and release excess amino acids lets one assume that export genes or proteins specific for the amino acid transport do not exist, but that the amino acids are excreted by way of other export systems.

The export systems known so far export poisonous metal ions, toxic antibiotica and higher molecular toxins. These export systems are relatively complex in their structure. Generally, membrane proteins of the cytoplasmic membrane are involved which however cause only a partial reaction of the export so that presumably additional extra cytoplasmic support proteins are needed for the transport (Dink, T. et al., A family of large molecules across the outer membranes of gram-negative bacteria., J. Bacteriol. 1994, 176: 3825-3831). Furthermore, it is known that, with the sec-dependent export system for extra-cellular proteins, at least six different protein components are essential for the export. This state-of-the-art suggests that also the systems, which are responsible for the export of amino acids, but which are not known so far comprise several protein components or respectively, several genes are responsible for the export of amino acids. A hint in this direction could be the various mutants which are defective in the lysine export as described by Vrylic et al.,(J. Bacteriol (1995) 177:4021-4027).

It has now been found surprisingly that only a single specific gene is responsible for the export of amino acids so that, in accordance with the invention, for the first time a method for the microbial manufacture of amino acids is provided wherein clearly the export gene expression and/or the export carrier activity of a microorganism producing amino acids is increased. The increased export expression or respectively, activity of the export carrier resulting from this process leads to an increased secretion rate so that the export of the respective amino acid is increased. The microorganisms so

modified also accumulate an increased part of the respective amino acid in the culture medium.

For an increase in the export carrier activity especially the endogenic activity of an amino acid producing microorganism is increased. An increase of the enzyme activity can be obtained for example by an increased substrate consumption achieved by changing the catalytic center or by eliminating the effects of enzyme inhibitors. An increased enzyme activity can also be caused by an increased enzyme synthesis for example by gene amplification or by eliminating factors which inhibit the enzyme biosynthesis. The endogene export activity is increased preferably by mutation of the endogenic export gene. Such mutations can be generated either in an uncontrolled manner in accordance with classic methods as for example by UV irradiation or by mutation causing chemicals or in a controlled manner by gene-technological methods such as deletion(s) insertion(s) and/or nucleotide exchange(s).

The export gene expression is increased by increasing the number of gene copies and/or by increasing regulatory factors which positively affect the export gene expression. For example, a strengthening of regulatory elements takes place preferably on the transcription level by increasing particularly the transcription signals. This can be accomplished for example in that, by changing the promoter sequence arranged before the structure gene, the effectiveness of the promoter is increased or by completely replacing the promoter by more effective promoters. An amplification of the transcription can also be achieved by accordingly influencing a regulator gene assigned to the export gene as will be explained further below. On the other hand, an amplification of the translation is also possible, for example, by improving the stability of the m-RNA.

To increase the number of gene copies the export gene is installed in a gene construct or, respectively, in a vector,

preferably, a vector with a small number of copies. The gene construct includes regulatory gene sequences, which are specifically assigned to the export gene, preferably such sequences which reinforce the gene expression. The regulatory gene sequences comprise a nucleotide sequence which codes for the amino acid sequence given in table 1 or the allele variations thereof or respectively, a nucleotide sequence 954 to 82 according to table 2 or a DNA sequence which is effective essentially in the same manner.

Allele variations or, respectively, equally effective DNA sequences comprise particularly functional derivatives which can be obtained by deletion(s) insertion(s) and/or substitution(s) of nucleotides of corresponding sequences, wherein however the regulator protein activity or function is retained or even increased. In this way, the effectiveness of the interaction of the regulatory protein to the DNA of the export gene to be regulated can be influenced by mutating the regulatory gene sequence such that the transcription is strengthened and, consequently, the gene expression is increased. In addition, also so-called enhancers may be assigned to the export gene as regulatory sequences whereby, via an improved correlation between RNA polymerase and DNA, also the export gene expression is increased.

For the insertion of the export gene into a gene construct, the gene is preferably isolated from a microorganism strain of the type Corynebacterium and, with the gene construct including the export gene, a microorganism strain, especially Corynebacterium, producing the respective amino acid is transformed. The isolation and transformation of the respective transport gene occurs according to the usual methods. If a transport gene is isolated and cloned from Corynebacterium then for example, the method of homologuous complementation of an export defective mutant is suitable (J.Bacteriol. (1995)177:

4021-4027). If a direct cloning of the structure gene is not possible vector sequences may first be inserted into the transport gene whereupon it is isolated by way of "plasmid rescue" in the form of inactive fragments. For the process according to the invention genes from the C. glutamicum ATCC 13032 or C. glutamicum ssp. flavum 14067 or also, C. glutamicum ssp. lacto fermentum ATCC 13869 are particularly suitable. The isolation of the genes and their in-vitro recombination with known vectors (Appl. Env. Microbial (1989)55: 684-688; Gene 102(1991)93-98) is followed by the transformation into the amino acid producing strains by electroporation (Liebl et al. (1989)FEMS Microbiol Lett. 65; 299-304) or$_p$ conjugation (Schäfer et al. (1990) J. Bacteriol. 172:1663-1666). For the transfer, preferably vectors with low numbers of copies are used. As host cells, preferably such amino acid producers are used which are deregulated in the synthesis of the respective amino acids and/or which have an increased availability of central metabolism metabolites.

After isolation, export genes with nucleotide sequences can be obtained which code for the amino acid sequences given in table 3 or for their allele variations or, respectively, which include the nucleotide sequence of 1016 to 1725 according to table 2 or a DNA sequence which is effective essentially in the same way. Also here, allele variations or equally effective DNA sequences include particularly functional derivatives in the sense indicated above for the regulatory sequences. These export genes are preferably used in the process according to the invention.

One or several DNA sequences can be connected to the export gene with or without attached promoter or respectively, with or without associated regulator gene, so that the gene is included in a gene structure.

By cloning of export genes, plasmids or, respectively,

vectors can be obtained which contain the export gene and which, as already mentioned, are suitable for the transformation of an amino acid producer. The cells obtained by transformation which are mainly transformed cells from Corynebacterium, contain the gene in reproducible form, that is, with additional copies on the chromosome wherein the gene copies are integrated at any point of the genome by homologous recombination and/or on a plasmid or respectively, vector.

A multitude of sequences is known which code for membrane proteins of unknown function. By providing in accordance with the invention export genes such as the export gene with the nucleotide sequence of nucleotide 10165 to 1725 in accordance with table 2 or respectively, the corresponding export proteins for example that with the amino acid sequence according to table 1, it is now possible to identify by sequence comparison membrane proteins, whose function is the transport of amino acids. The export gene identified in this way can subsequently be used to improve the amino acid production in accordance with the process of the invention.

The membrane proteins known from the state-of-the-art generally include 12, some also only 4 transmembrane helices. However, it has now been found surprisingly that the membrane proteins responsible or suitable for the export of amino acids include 6 transmembrane helices (see for example, the amino acid sequence of an export protein listed in the table 3, wherein the 6 transmembrane areas have been highlighted by underlining). Consequently, there is a new class of membrane proteins present which has not yet been described.

Examples:

a) Cloning of an export gene and cloning of a regulator of Corynebacterium glutamicum.

Chromosomal DNA from C. glutamicum R127 (FEMS Microbiol lett. (1989)65:299-304) was isolated as described by Scharzer

et al. (Bio/Technology (1990) 9:84-87). The DNA was then split with the restriction enzyme Sau3A and separated by saccharose gradient centrifugation as described in Sambrook et al. (Molecular cloning, A laboratory manual (1989) Cold Spring Harbour Laboratory Press). The various fractions were analyzed gel electrophoretically with respect to their size and the fraction with a fragment size of about 6 - 10kb was used for the ligation with the vector pJC1. In addition, the vector pJC1 was linearized with BamHI and dephosphorylized. Five ng thereof was ligated with 20ng of the chromosomal 6-10 kb fragments. With the whole ligation preparation, the export defective mutant NA8 (J. Bacterol. (1995)177:4021-4027) was transformed by electroporation (FEMS Microbiol Lett(1989)65:299 - 304). The transformants were selected for LBHIS(PEMS Microbiol. Lett. (1989)65:299-304) with 15µg kanamycin per ml. These transformants were subjected to extensive plasmid analyses in that 200 of the altogether 4500 clones obtained were individually cultivated and their plasmid content and size was determined. On average, about half of the kanamycin-resistant clones carried a recombinant plasmid with an insert of the average size of 8kb. This provides for a probability of 0.96 for the presence of any gene of C. glutamicum in the established gene bank. The 4500 obtained transformants were all individually checked for renewed presence of lysinee secretion. For this purpose, the system described by Vrljic for the induction of the L-lysinee excretion in Corynebacterium glutamicum was utilized (J. Bacteriol (1995) 177:4021-4027). For this purpose, so-called minimal-medium-indicator plates were prepared, which contained per liter 20g $(NH_4)_2SO_4$, 5g uric acid, 1g KH_2PO_4, 1 g K_2HPO_4, 0.25g $MgSO_4x7H_2O$, 42 g morpholino propane sulfonic acid, 1ml $CaCl_2$ (1g/100ml), 750 ml dest., 1 ml Cg trace salts, 1 ml biotin (20µg/100l), pH7, 4% glucose, 1.8mg protocatechuic acid, 1 mg $FeSO_4$ x 7 H_2O, 1 mg $MnSO_4$ x H_2O, 0.1 mg $ZnSO_4$ x $7H_2O$,

0.02mg $CuSO_4$, 0.002mg $NiCl_2 \times 6H_2O$, 20 g agar-agar, as well as 10^7 cells/ml of the lysine-auxotrophene C. glutamicum mutant 49/3. The original 4500 transformants were all individually pinned, by toothpicks onto the indicator plates with, in each case, a check of the original non-excretor NA8 (J.Bacteriol (1995)177:4021-4027) and the original strain R127. At the same time, always 2 plates were inoculated of which only one contained additionally 5mM L-methionine in order to induce the lysine excretion in this way. The indicator plates were incubated at 30°C and examined after 15, 24 and 48 hours. In this way, altogether 29 clones were obtained which showed on the indicator plate provided with methionine a growth court by the indicator strain 49/3. The clones were examined individually and then again as described above, for reestablishment of the growth court. In this way, the two clones NA8 pMV8-5-24 and NA8 pMV6-3 were obtained which had again received the capability to excrete lysine.

From these clones, plasmid preparations were performed as described in Schwarzer et al. (Bio/Technology (1990)9; 84-87). By retransformation in NA8, the plasmid-connected effect of the excretion of L-lysine was confirmed. Both plasmids were subjected to a restriction analysis. Plasmid pMV8-5-24 carries an insert of 8.3 kb, and pMV6-3 one of 9.5 kb. The physical charter of the inserts is shown in Fig. 1.

b) Subcloning of an DNA fragment which reconstitutes the lysine export.

From the insert of the plasmid pMV6-3 individual subclones were prepared utilizing the restriction severing point as determined. In this way, the 3.7 kb XhoI-SalI-fragment, the 2.3 kb BamHI-fragment and the 7.2 kb BamHI fragment were ligated with the correspondingly severed and treated vector pJC1 (Mol Gen. Genet.(1990)220: 478-480). With the ligation products C. glutamicum NA8 was directly transformed, the transformants were

tested for having the lysine excretion properties and the presence of the subclone was confirmed by plasmid preparation and restriction analysis. In this way, the strain with plasmid pMV2-3 (Fig. 1) was obtained as smallest subclone. This fragment resulting in lysine export contains as insert the 2.3kb Bam fragment from pMV6-3.

c) Sequence of the lysine export gene lys E and its regulators lysG.

The nucleotide sequence of the 2.3kb BamH1 fragment was performed according to the dideoxy-chain termination method of Sanger et al. (Proc. Natl. Acad. Sci USA(1977) 74:5463-5467) and the sequencing reaction with the Auto Read Sequencing kit from Pharamcia (Uppsala, Sweden). The electrophoretic analysis occurs with the automatic laser-fluorescence DNA sequencing apparatus (A.L.F) from Pharmacia-LKB(Piscataway, NJ, USA). The nucleotide sequence obtained was analyzed by the program packet HUSAR (Release 3.0) of the German Cancer Research Center (Heidelberg). The nucleotide sequence and the result of the analysis is presented in Fig. 2. The analysis results in two fully open reading frames (ORF) on the sequenced DNA piece. ORF1 codes for a protein with a length of 236 amino acids, OFR2 codes for a protein with a length of 290 amino acids. The protein derived from ORF1 includes an accumulation of hydrophobic amino acids as they are characteristic for membrane-embedded proteins. The detailed analysis of the distribution of the hydrophobic and hydrophilic amino acids by the programs PHD.HTM (Protein Science(1995)4:521-533) is shown in table 3. It is apparent therefrom that the protein contains six hydrophobic helix areas which extend through the membrane. Consequently, this protein is the searched for exporter of the amino acid L-lysine. The corresponding gene will therefore be designated below as lysE. In table 2, it is marked accordingly. ORF2 is transcribed in a direction opposite to ORF1. The sequence

analysis shows that ORF2 has a high identity with regulator genes which are combined as a single family (Ann Rev Microbiol(1993) 597-626). Genes of this family regulate the expression processes of the various genes involved in catabolic or anabolic processes in a positive way. For this reason, ORF2 will below be designated as lysG (Govern=regulating). Because of the coordination and because lysE could be cloned (see a)) and subcloned (see b)) together with lysG, lysG is regulator of lysE and consequently also participates in the lysine export. The gene lysG and the amino acid sequence derived therefrom are also shown in table 2 and, respectively, table 1.

d) Identification of an unknown membrane protein from Escherichia coli by sequence comparison.

With the established sequences according to table 3 already existing sequence banks can be searched in order to assign the proteins derived in this way from sequenced areas a certain function. Correspondingly, the amino acid sequence of the lysine exporters consisting of C. glutamicum were compared with derivated protein sequences of all the DNA sequences deposited there utilizing the program packet HUSAR (Release 3.0) of the German Cancer Research Center (Heidelberg). A high homology of 39.3% identical amino acids and 64.9% similar amino acids was found to a single sequence of so far unknown function of E.coli.

The comparison is shown in Fig. 2. The open read frame of E. coli so far not characterized is consequently identified by way of this process as an amino export gene.

e) Increased export of intracellularly accumulated L-lysine.

The strain C. glutamicum NA8 (J. Bacteriol(1995) 177: 4021-4027 was transformed with plasmid pMV2-3 and the L-lysine excretion of the strains was compared. For this purpose, NA8 and NA8pMV2-3 in complex medium were utilized as described in

Vrljic et al. (J. Bacteriol (1995)177:4021-40277) and the fermentation medium CGXII (Bacteriol (1993)175:5595-5603 were each separately inoculated. The medium additionally contained 5mM L-methionin in order to induce the intracellular L-lysine biosynthesis. After cultivation for 24 hours at 30°C on a rotary vibrator at 140 rpm, the cell internal and external L-lysine determinations were performed. For the cell-internal determination silicon oil centrifugations were performed (Methods Enzymology LV(1979) 547-567); the determination of the amino acids occurred by high pressure liquid chromatography (J. Chromat (1983) 266:471-482). These determinations were performed at different times as indicated in Fig. 3. In accordance with the process used the retained cell internal L-lysine is excreted also by pMV2-3 to a greater degree and is accumulated. Accordingly, also the cell internally present L-lysine is greatly reduced. Consequently, the utilization of the newly discovered and described exporter represents a process for greatly improving the L-lysine production.

f) Increased accumulation of L-lysine by lysE or LysEG.

From the subclone pMV2-3 which contains the sequenced 2374bp Bam HI-fragment in pJCI (see figure 1), the lysE carrying 1173 bpPvuII fragment was ligated in pZ1 (Appl. Env. Microbiol(1989)55:684-688) according to the sequence information and in this way, the plasmid plysE was obtained. This plasmid as well as the lysE lysG carrying plasmid pMV2-3 was introduced into C. glutamicum strain d by electroporation wherein the chromosomal areas were deleted. The obtained strains C. glutamicum d pMV2-3, C. glutamicum d plysE; C. glutamicum pJC1 were, as described under e) precultivated on a complex medium, then cultivated in production minimal medium CGx11 together with 4% glucose and 5mM l-methionin and samples were taken to determine the accumulated lysine. As apparent from Fig. 4 with lysE lysG an increase of the lysine accumulation with respect

to a control sample is achieved. With plysE an extraordinarily increased accumulation of from 4.8 to 13.2 mM L-lysine is achieved with this method.

LEGENDS OF THE TABLES AND FIGURES

Table 1: The amino acid sequence of the lysine exporter regulator from Corynebacterium glutamicum with the helix-turn-helix motive typical for DNA-binding proteins.

Table 2(three pages): The nucleotide sequence of C. glutamicum coding for the lysine exporter and lysine export regulators.

Table 3: the amino acid sequence of the lysine exporter from Corynebacterium glutamicum with the identified transmembrane helices TMH1 to TMH6.

Figure 1: the fragments in pMV6-3 and pMV8-5-24 obtained by the cloning which cause the lysine secretion and the subclone pMV2-3 made from pMV6-3, which also causes the lysine secretion and which was sequenced. B, BamH1; Sm, SmaI; Se, SacI; Sl, Sal I,II, HindII; X, XhoI.

Figure 2: Comparison of the derivated amino acid sequence of LysE from C. glutamicum (above), with a gene product of so far unknown function from Escherichi coli (below), which is identified thereby as export carrier.

Fig. 3: Increased lysine export by pMV2-3 with C. glutamicum NA8. On top, the control with low excretion and cell-internal backup of lysine up to about 150mM. Below, the high secretion caused by pMV2-3 with cell internally only small backup of about 30mM.

Figure 4: the increase of the lysine accumulation in C. glutamicum by lys E lys G(pMV2-3) (middle curve), and the accumulation caused by lysE(plysE) (upper curve).

EDITORIAL NOTE

No: 19218/97

The following five pages are unnumbered

(Table 1 to Table 3)

1 MNPIQLDTLL SIIDEGSFEG ASLALSISPS AVSQRVKALE HHVGRVLVSR

Helix-Turn-Helix-Motiv

51 TQPAKATEAG EVLVQAARKM VLLQAETKAQ LSGRLAEIPL TIAINADSLS

101 TWFPPVFNEV ASWGGATLTL RLEDEAHTLS LLRRGDVLGA VTREANPVAG

151 CEVVELGTMR HLAIATPSLR DAYMVDGKLD WAAMPVLRFG PKDVLQDRDL

201 DGRVDGPVGR RRVSIVPSAE GFGEAIRRGL GWGLLPETQA APMLKAGEVI

251 LLDEIPIDTP MYWQRWRLES RSLARLTDAV VDAAIEGLRP

Table 1

1 MNPIQLDTLL SIIDEGSFEG ASLALSISPS AVSQRVKALE HHVGRVLVSR

Helix-Turn-Helix-Motiv

51 TQPAKATEAG EVLVQAARKM VLLQAETKAQ LSGRLAEIPL TIAINADSLS

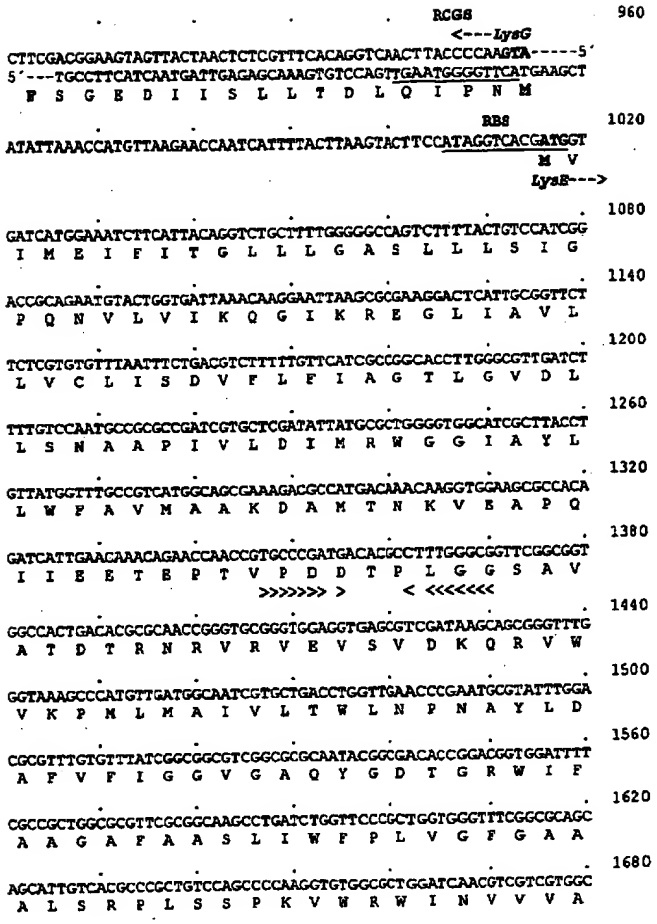
101 TWFPPVFNEV ASWGGATLTL RLEDEAHTLS LLRRGDVLGA VTREANPVAG

151 CEVVELGTMR HLAIATPSLR DAYMVDGKLD WAAMPVLRFG PKDVLQDRDL

201 DGRVDGPVGR RRVSIVPSAE GFGEAIRRGL GWGLLPETQA APMLKAGEVI

251 LLDEIPIDTP MYWQRWRLES RSLARLTDAV VDAAIEGLRP

Table 1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<---LysG
960
CTTCGACGGAAGTAGTTACTAACTCTCGTTTCACAGGTCAACTTACCCCAAGTA-----5'
5'---TGCCTTCATCAATGATTGAGAGCAAAGTGTCCAGTTGAATGGGGTTCATGAAGCT
F S G E D I I S L L T D L Q I P N M
RBS
1020
ATATTAAACCATGTTAAGAACCAATCATTTTACTTAAGTACTTCCATAGGTCACGATGGT
M V
LysE--->
1080
GATCATGGAAATCTTCATTACAGGTCTGCTTTTGGGGGCCAGTCTTTTACTGTCCATCGG
I M E I F I T G L L L G A S L L L S I G
1140
ACCGCAGAATGTACTGGTGATTAAACAAGGAATTAAGCGCGAAGGACTCATTGCGGTTCT
P Q N V L V I K Q G I K R E G L I A V L
1200
TCTCGTGTGTTTAATTTCTGACGTCTTTTTGTTCATCGCCGGCACCTTGGGCGTTGATCT
L V C L I S D V F L F I A G T L G V D L
1260
TTTGTCCAATGCCGCGCCGATCGTGCTCGATATTATGCGCTGGGGTGGCATCGCTTACCT
L S N A A P I V L D I M R W G G I A Y L
1320
GTTATGGTTTGCCGTCATGGCAGCGAAAGACGCCATGACAAACAAGGTGGAAGCGCCACA
L W F A V M A A K D A M T N K V E A P Q
1380
GATCATTGAACAAACAGAACCAACCGTGCCCGATGACACGCCTTTGGGCGGTTCGGCGGT
I I E E T E P T V P D D T P L G G S A V
>>>>>>> > < <<<<<<<
1440
GGCCACTGACACGCGCAACCGGGTGCGGGTGGAGGTGAGCGTCGATAAGCAGCGGGTTTG
A T D T R N R V R V E V S V D K Q R V W
1500
GGTAAAGCCCATGTTGATGGCAATCGTGCTGACCTGGTTGAACCCGAATGCGTATTTGGA
V K P M L M A I V L T W L N P N A Y L D
1560
CGCGTTTGTGTTTATCGGCGGCGTCGGCGCGCAATACGGCGACACCGGACGGTGGATTTT
A F V F I G G V G A Q Y G D T G R W I F
1620
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A A G A F A A S L I W F P L V G F G A A
1680
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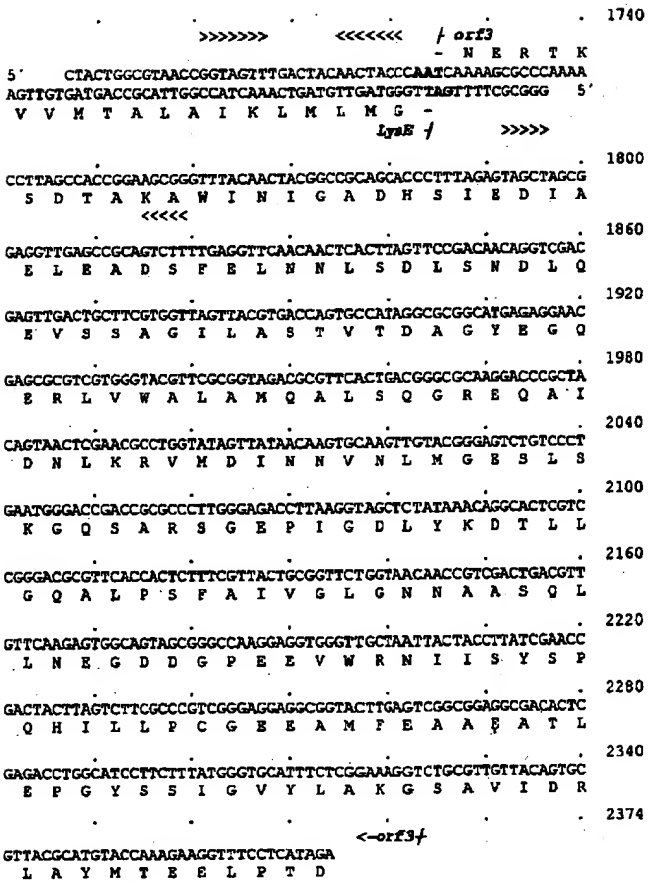


1 MVIMEIFITG LLLGASLLLS IGPQNVLVIK QGIKREGLIA VLLVCLISDV

TMH1 TMH2

51 FLFIAGTLGV DLLSNAAPIV LDIMRWGGIA YLLWFAVMAA KDAMTNKVEA

TMH3

101 PQIIEETEPT VPDDTPLGGS AVATDTRNRV RVEVSVDKQR VWVKPMLMAI

151 VLTWLNPNAY LDAFVFIGGV GAQYGDTGRW IFAAGAFAAS LIWFPLVGFG

TMH4 TMH5

201 AAALSRPLSS PKVWRWINVV VAVVMTALAI KLMLMG

TMH6

Table 3

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Process for the microbial production of amino acids wherein a bacterial microorganism is modified such that the activity of the export carrier which is specific for the corresponding amino acid and which is encoded by a single export gene is increased and/or such that the expression of the single export gene specific for the corresponding amino acid of a bacterial microorganism producing the respective amino acid is increased.

2. Process according to claim 1, characterized in that the endogenous export carrier activity of the microorganism is increased.

3. Process according to claim 2, characterized in that by mutation of the endogenous export gene a carrier with higher export activity is generated.

4. Process according to one of the claims 1 to 3, characterized in that the gene expression of the export carrier is increased by increasing the number of gene copies.

5. Process according to claim 4, characterized in that to increase the number of copies the export gene is installed in a gene construct.

6. Process according to claim 5, characterized in that the export gene is installed in a vector with a low number of copies.

7. Process according to claim 5 or 6, characterized in that the export gene is installed in a gene construct which includes regulatory gene sequences assigned to the export gene.

8. Process according to claim 7, characterized in that the regulatory gene sequence includes a nucleotide sequence coding for the amino acid sequence given in table 1 and the allele variations thereof.

9. Process according to claim 9, characterized in that the

regulatory gene sequence includes a nucleotide sequence of nucleotide sequence of nucleotide 954 to 82 according to table 2 or a DNA sequence effective essentially in the same way.

10. Process according to one of the claims 5 to 9, characterized in that a microorganism producing the respective amino acid is transformed with the gene construct including the export gene.

11. Process according to claim 10, characterized in that a microorganism of the type Corynebacterium is transformed with the gene construct including the export gene.

12. Process according to claim 10 or 11, characterized in that for the transformation a microorganism is utilized in which the enzymes which participate in the synthesis of the corresponding amino acids are deregulated.

13. Process according to one of the claims 10 to 12, characterized in that for the transformation a microorganism is utilized which contains an increased part of the central metabolism metabolites.

14. Process according to one of claims 4 to 13, characterized in that the export gene is isolated from a microorganism strain of the type Corynebacterium.

15. Process according to one of the preceding claims, characterized in that the export gene sequence is identified by comparison with the sequence of an already known export gene.

16. Process according to claim 15, characterized in that that the amino acid sequence derived from the export gene sequence to be identified is compared with the amino acid sequence given in table 3 or the allele variation thereof.

17. Process according to one of the preceding claims, characterized in that the export gene expression is increased by amplifying the transcription signals.

18. Process according to one of the preceding claims, characterized in that as export gene, a gene with a nucleotide

sequence coding for the amino acid sequence given in table 3 and the allele variations thereof is utilized.

19. Process according to claim 18, characterized in that as export gene a gene with the nucleotide sequence of nucleotide 1016 to 1725 according to table 2 or a DNA sequence with essentially the same effects is utilized.

20. Process according to one of the preceding claims for the manufacture of L-lysine.

21. A single isolated or modified bacterial export gene coding for an amino acid export carrier.

22. Export gene according to claim 21 with a nucleotide sequence coding for an amino sequence given in table 3 or the allele variation thereof.

23. Export gene according to claim 22 with the nucleotide sequence of nucleotide 1016 to 1725 according to table 2 or a DNA sequence with essentially the same effects.

24. Export gene according to one of the claims 21 to 23 with regulatory gene sequences assigned thereto.

25. Export gene according to claim 24, characterized in that the regulating gene sequence includes a nucleotide sequence coding for the amino sequence given in table 1 and the allele variations thereof.

26. Export gene according to claim 25, characterized in that the regulating gene sequence includes a nucleotide sequence of nucleotide 954 to 82 according to table 2 or a DNA sequence effective essentially in the same way.

27. An isolated or modified regulator gene suitable for the regulation of an export gene coding for an amino acid and export carrier, including a nucleotide sequence coding for the amino sequence given in table 1 and the allele variations thereof.

28. Regulator gene according to claim 27 with the nucleotide sequence of nucleotide 954 to 82 according to table 2 or a DNA sequence effective essentially in the same way.

29. Gene structure containing an export gene according to

one of claims 21 to 24.

30. Gene structure including a regulatory gene sequence according to claim 27 or 28.

31. Vector including an export gene according to one of claims 21 to 26 or a gene structure according to claim 29.

32. Vector according to claim 31 with a low number of copies.

33. Vector including a regulatory gene sequence according to claim 27 or 28 or a gene structure according to claim 30.

34. Transformed cell including, in a replicable form, an export gene according to one of the claims 21 to 26 or a gene structure according to claim 29.

35. Transformed cell according to claim 34 including a vector according to claim 31 or 32.

36. Transformed cell according to claim 34 or 35, characterized in that it belongs to the type Corynebacterium.

37. Transformed cell according to one of claims 34 to 36, characterized in that in this cell the enzymes of the amino acid, which participate in the synthesis, are deregulated and wherein the amino acid is removed from the cell by way of the export carrier for which the export gene, which was transferred into the transformed cell, codes.

38. Transformed cell according to one of claims 34 to 37, characterized in that the cell includes an increased proportion of central metabolism metabolites.

39. Transformed cell including, in replicable form, a regulatory gene sequence according to claim 27 or 28 or a gene structure according to claim 30.

40. Transformed cell according to claim 39, including a vector according to claim 33.

41. An isolated or modified bacterial membrane protein specific for the export of amino acids comprising 6 transmembrane helices.

42. Membrane protein according to claim 41, including the

amino acid sequence given in table 3 wherein table 3 is part of this claim.

43. Use of a single isolated or modified bacterial export gene encoding an amino acid export carrier for increasing the amino acid production of micro-organisms.

44. Use according to claim 43, characterized in that a mutated export gene, which codes for an enzyme with increased export carrier activity is utilized.

45. Use according to claim 43 or 44, characterized in that the amino acid producing microorganism is transformed with a gene construct which includes an export gene.

46. Use according to claim 45, characterized in that the gene construct additionally carries regulatory gene sequences.

47. Use according to one of the claims 43 to 46, characterized in that an export gene from Corynebacterium is utilized.

48. Use according to one of claims 43 to 47, characterized in that Corynebacterium is used as amino acid producing microorganism.

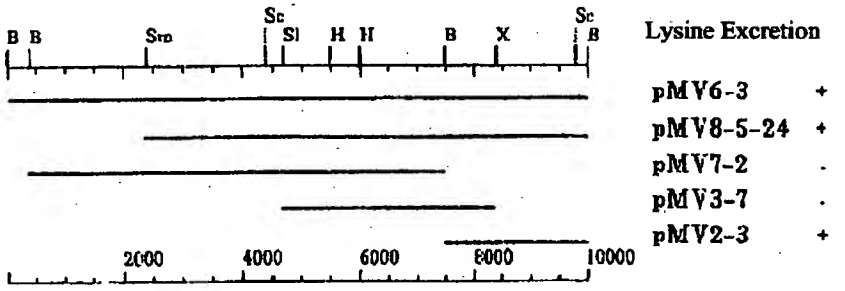
49. A process according to any one of claims 1 to 20 or an export gene according to any one of claims 21 to 26 or a regulator gene according to any one of claims 27 and 28 or a gene structure according to claims 29 or 30 or a vector according to any one of claims 31 to 33 or a transformed cell according to any one of claims 34 to 40 or a membrane protein according to any one of claims 41 or 42 or a use according to any one of claims 43 to 48 substantially as hereinbefore described with reference to the Figures and/or Examples.

DATED this 25th day of JULY, 2000

Forschungszentrum Julich GmbH

DAVIES COLLISON CAVE

Patent Attorneys for the Applicant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Sma
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Sl
H H
B
X
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B
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4000
6000
8000
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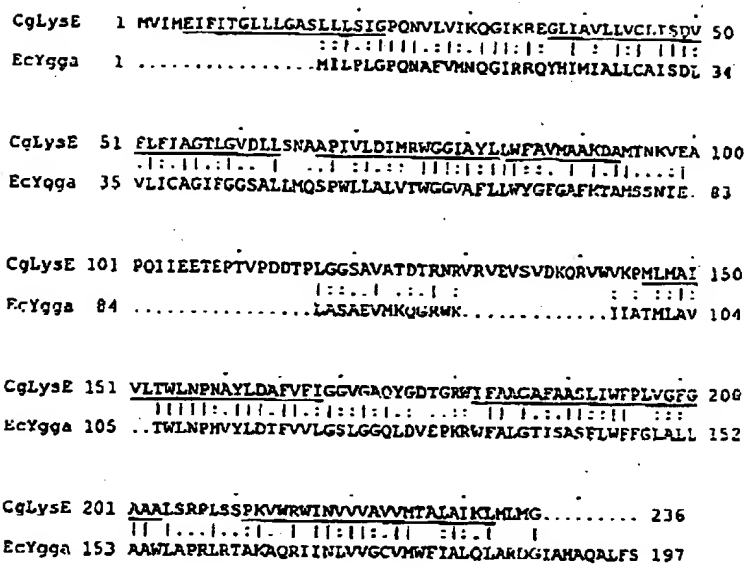
pMV8-5-24 +
pMV7-2 -
pMV3-7 -
pMV2-3 +

Figure 1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CgLysE 51 FLFIAGTLGVDLLSNAAPIVLDIMRWGGIAYLLWFAVMAAKDAMTNKVEA 100
EcYgga 35 VLICAGIFGGSALLMQSPWLLALVTWGGVAFLLWYGFGAFKTAMSSNIE. 83
CgLysE 101 PQIIEETEPTVPDDTPLGGSAVATDTRNRVRVEVSVDKQRVWVKPMLMAI 150
EcYgga 84 .....LASAEVMKQGRWK.....IIATMLAV 104
CgLysE 151 VLTWLNPNAYLDAFVFIGGVGAQYGDTGRWIFAAGAFAASLIWFPLVGFG 200
EcYgga 105 ..TWLNPHVYLDTFVVLGSLGGQLDVEPKRWFALGTISASFLWFFGLALL 152
CgLysE 201 AAALSRPLSSPKVWRWINVVVAVVMTALAIKLMLMG..... 236
EcYgga 153 AAWLAPRLRTAKAQRIINLVVGCVMWFIALQLARDGIAHAQALFS 197

Figure 2

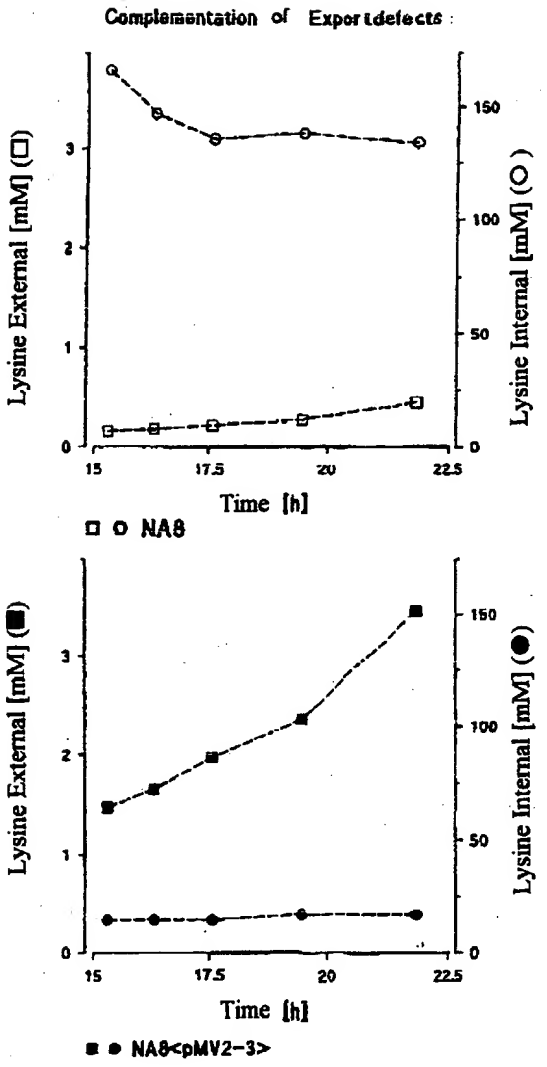
Complementation of Export defects
Lysine External [mM] (□)
Lysine Internal [mM] (○)
Time [h]
□ ○ NA8
Lysine External [mM] (■)
Lysine Internal [mM] (●)
Time [h]
■ ● NA8<pMV2-3>

Figure 3

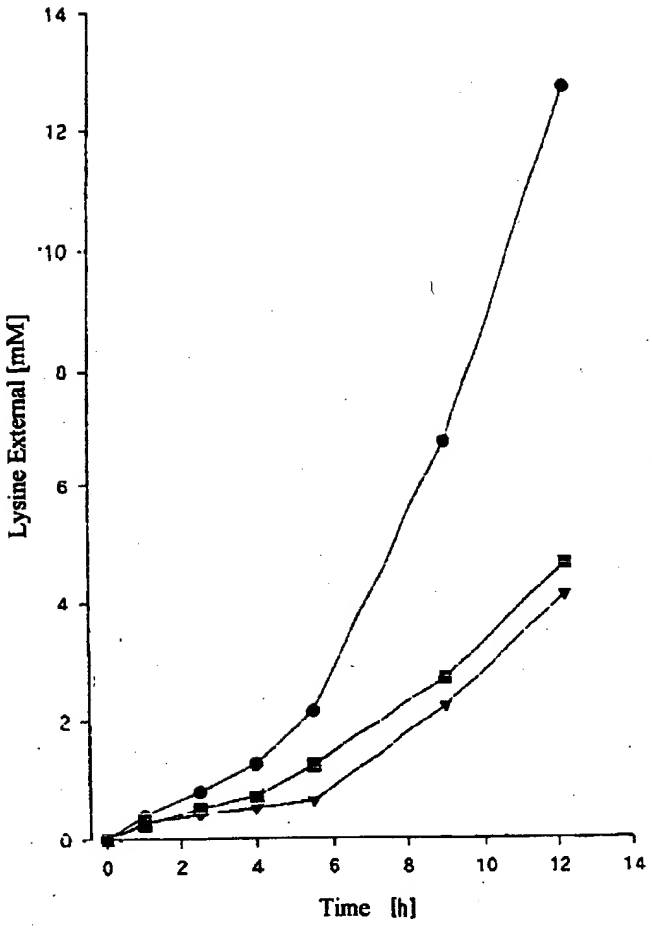

Figure 4